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ORIGINAL ARTICLES

ORGANISMS ASSOCIATED WITH SUGARCANE MOSAIC AND THEIR RELATION TO THE MOSAIC VIRUS

BY

S. V. DESAI, B. Sc., Ph.D., D.I.C.,

ERRATA.

Page 403, line 8 from bottom, *delete* hyphen between the words "were" and "stem-sterilised".

Page 407, head line, *read* "PARASITISM" *for* "PARSAITISM".

Page 409, head line, *read* "PARASITISM" *for* "FARASITISM".

Page 409, line 3 from bottom, *insert* hyphen after the word "No".

Page 411, head line, *read* "OF" *for* "GF".

Page 413, head line, *read* "PARASITISM" *for* "PARASITISN".

Page 418, head line, *read* "OF" *for* "F".

with the virus whatever the latter may be. However, inoculation experiments with these organisms were unsuccessful in reproducing the disease.

Bewley [1931] advanced a suggestion from his observation of constant association of pleomorphic bacteria with tomato mosaic that the virus was a kind of bacteriophage living on the bacteria and parasitizing the plant under suitable conditions. Swezy and Severin [1930] reported the association of rickettsia-like micro-organisms with curly top of sugar beet. They were unable to differentiate on morphological data between the two types of organisms which they found—but one type was found to pass the Berk feld filter while the other did not. Filterable ones were found only in the infective insect vectors, the others occurring both in healthy and infective insects.

Takahashi and Rawlins [1932] have put forward evidence of the existence of minute rod-shaped bodies in sap containing virus by examining the clarified juice of tobacco mosaic by polarised light. Melhus [1922], Bouquet [1916] and Smith and Bouquet [1915] have associated bacteria with the causative agents of

plant virus diseases. Brehmer and Barner [1930] and Brehmer [1931] described yellowish green, oval, amorphous bodies which they consider are organisms belonging to a hitherto unknown systematic group of the Archimycetes allied to Plasmodiophoracæ.

Desai [1933] evolved a method of cultivation of the virus principle *in vitro* and gave evidence of virus principle being related to forms of bacteria usually found associated with tomato mosaic and photographed the minute flagellate granules after mordanting them and staining with flagella stains.

The present work was undertaken to find out whether similar organisms were associated with sugarcane mosaic, and if so to find out their relationship to the mosaic virus.

II. Isolation

A. By tissue implantations

The first method tried to obtain cultures of bacteria associated with sugarcane mosaic was by tissue implantations. For this purpose the stem of sugarcane having mosaic disease was thoroughly sterilized by repeated washing with mercuric chloride and alcohol. A sterile cork borer was then inserted in the internodes near the growing point and an inch of the plug was quickly transferred to an agar slant directly from the cork-borer. The cork-borer was plugged with cotton at the top end to prevent infection from outside.

The media generally used were sugarcane extract broth and agar of the following composition :—

Broth	Agar
0·5 grm. dipotassium phosphate	
0·2 grm. magnesium sulphate	
3·0 grms. Marmite	1.5 per cent agar was added for agar medium.
1·0 grm. Mannite or cane sugar	
1000 c.c. of sugarcane leaf extract	
The extract was prepared by steaming 200 grms. of sugarcane leaves in 1000 c.c. of water for 30 minutes and filtering.	

After adjusting the pH to 7.8 the media were sterilized at 120°C. for 30 minutes.

The culture tubes were incubated at 30°C. for long periods after sealing them with paraffin wax. In some cases fungi and bacteria were found growing abundantly in a couple of days time. Some tubes remained sterile for more than six months by which time the agar had usually dried up, and in some tubes thin growth of bacteria developed after a month or so. These tubes were still moist with water of condensation while the tubes which ultimately remained sterile for six months were dry and without any water of condensation at this stage though sealed up with paraffin wax.

Moisture appeared to influence the growth. Sub-cultures from these bacterial growths were difficult to cultivate on agar and generally no growth was visible in the transferred slants. Plates poured were also devoid of visible colonies but occasionally very minute colonies were noticed. The colonies were imperceptible with the naked eye but could be viewed under a low magnification.

Visible growth was obtained only after sub-culturing such isolated colonies six to seven times. Usually after 10 transfers typical, easily cultivable bacterial growth was established. The cultures by this method were obtainable in 60 per cent of cases tried, in 30 per cent of cases contaminations occurred, while in 10 per cent of the cases the tubes remained sterile till they dried up.

B. By broth transfers

Methods of serial broth transfers have been recommended by Hadley [1931] to demonstrate the presence of the filterable forms of bacteria. Sugarcane extract broth as previously described was ordinarily used.

The juice of mosaic leaves 0.2 to 0.5 c. c., filtered through L_3* filter candle, was introduced in broth tubes and incubated for 5 days. No visible growth of any organism was found in the tubes. These tubes were filtered through a sterile L_3 filter candle and 0.2 to 0.5 c.c. of the filtrate put in fresh series of broth tubes. The process of filtration and transfer to fresh series of broth tubes was carried on until some visible sign of growth was observed in the culture tubes. This usually occurred after about 7 to 8 passages when a slight opalescence developed. The juice of the healthy sugarcane leaves under the same treatment failed to show similar phenomenon even after 20 such passages. The growth in broth at this stage if spread on agar failed to show visible colonies. The growth in serial passages after opalescence became progressively visible and after a passage or two became cultivable on the sugarcane extract agar. The growth on agar consisted of minute pin point colonies barely visible to the unaided eye. Usually after ten to eleven passages the growth became quite perceptible, with flakes ; and grew on agar in a uniform layer dotted with plaques, like a bacterial culture associated with its bacteriophage.

* Finer grades of filter candles L_3 to L_{13} were used and they too gave similar results.

The same type of growth was observed in 90 per cent of the serial passages carried out while in four per cent the culture tubes got contaminated and in six per cent of the cases the culture was not obtainable even after 20 serial passages. Simultaneously with each of the series with mosaic juice a similar series with juice of healthy leaves was carried out and in 94 per cent of the cases no growth was obtained, in two per cent of the cases contamination occurred and in four per cent of the cases the typical bacterial culture was obtained. It may be that the organisms were present in the plants which were considered healthy, the disease being present with masked symptoms, or in view of the constant presence in the laboratory of these organisms these four per cent of the cases may be better attributed to contamination with the organisms.

C. By ageing the leaf juice

The juice of the mosaic leaves was filtered through L_3 filter candle and incubated under sterile conditions after sealing in ampoules. The juice ordinarily remained clear for more than a year but some times after a couple of months slight opalescence developed which continued increasing till definite growth was visible. The sealed tubes were then broken and the juice implanted on agar surface and in broth. The agar showed some growth if the juice was cloudy, otherwise with slightly opalescent cultures no visible growth was observed on agar. In broth the growth was always obtained and appeared to be of the same type as obtained by successive passages previously described.

In twenty per cent of the sealed ampoules the growth described above developed and 80 per cent of the ampoules remained unchanged. But if serial transfers were made from these tubes in broth, very frequently similar growth was observed after three serial transfers.

III. Cultural behaviour

Cultivation of the organisms from the mosaic tissues was found to be difficult. Cultures were unaccountably lost and failed to show visible growth. Transfers of such unviable culture in broth often brought about slight opalescence but transfers from these showed no growth on various agar media. Gelatin media also proved equally unsuitable. Broth cultures, and their transfers also, often remained quite limpid with no visible growth. Various broth media were tried to fix upon the most suitable one. But there was little to choose as almost all media behaved similarly. Sugarcane extract broth with 0·2 per cent lithium chloride was found to be the best. The morphological forms in broth were mixed and variable. From very minute cocci to well sized rods were common when visible growth occurred. If only opalescence was present, no rods or cocci were

found but minute granule-like forms which could not be differentiated from stain particles or protein droplets of the media were prominent. Flagella staining at this stage did not differentiate these granules from usual media debris.

Attempts were made to study the cyclostages of the bacteria by repeated serial passages but no regular behaviour was observed as the visibly cultivable stage was found to be very short if transfers were continued in broth. Acidic and neutral culture media did not improve matters.

The relative lengths of various cyclostages of these organisms were compared with those of Shiga Bacillus (in which filterable forms and other cyclostages are well studied). This brought out the fact that these organisms were most stable in the virus cyclostage :—

TABLE I

	Bacterial cyclostage "R. & S." stage	Filterable cyclostage "G" forms	Virus cyclostage invisible growth stage
Organisms associated with mosaic virus .	4	8	88
Shiga Bacillus	78	20	2

Thus during the life cycle of 100 days these organisms had the bacterial cyclostage for only four days as against similar cyclostage of 78 days in Shiga cultures, filterable or "G" form cyclostage for eight days as against similar cyclostage of twenty days in Shiga cultures and invisible virus cyclostage of 88 days as against similar cyclostage of only two days in Shiga cultures. This accounted for the rapid transformation and unstable bacterial cyclostage of the virus organisms and their peculiar pleomorphic growth. It also explained why it was difficult to study the behaviour of the organisms by ordinary bacteriological technique and biochemical reactions.

Cultivation of the organisms on agar and other solid surface offered still greater difficulty than cultivation in broth. Virus or invisible cyclostage was not cultivable on agar and if continuous transfers were made by transferring water of condensation, though no growth was visible no growth developed and transfers to a series of broth tubes ultimately failed to reveal the presence of the

organisms. It was therefore concluded that cyclostage was unable to multiply on solid media, but with the change from " virus " cyclostage to opalescent forms and to " bacterial " stage the cultivation on agar was progressively successful. The cyclostage which gave opalescence in the broth media usually gave a growth of minute colonies invisible to the unaided eye but distinguishable under low power objective. The broth cultures which were distinctly cloudy gave uniform bacterial growth studded with minute round clear areas similar to bacteriophage plaque or minute colonies of different type than the ordinary smooth growth. These areas appeared to be devoid of any growth in the beginning but on ageing fine annular growth began to be visible. The growth was of different type to the surrounding one. Transfers from such plaques usually failed to grow on agar or any other solid media, in broth they sometimes produced opalescence, but mostly no change and rarely visible clouding typical of the organisms. The transfers from bacterial growth were easily cultured on solid as well as on broth media but sometimes the transfers appeared sterile without any clouding or colony formation, and in such cases it was extremely difficult to recover the culture from such culture tubes. If 24-hourly transfers were carried out the cultures soon got lost but with fortnightly or monthly transfers the culture could be maintained with slight difficulty. The cultures of minute colonies were not stable in that phase and more often failed to give growth on transfers in solid or broth media. Sometimes this phase or cyclostage was found to give the same type of growth on solid agar for some time and vanishing completely afterwards on further transfers. This cyclostage developed if Chamberland candle filtrates from the suspension of the old culture of the organisms were spread on agar surface and that was the only reliable source of this filter-passing visible cyclostage giving minute colonies. The culture of these minute colonies could be maintained as such for a few transfers after which the culture either failed to develop visibly or gave uniform growth composed of large colonies studded with minute plaques.

IV. Morphology and biochemical reactions

From the foregoing description it would be evident that the culture of these organisms was usually a mixture of different cyclostages but at the same time each cyclostage could be obtained in more or less pure form by suitable technique.

The life-history of the organisms may be subdivided into following well defined cyclostages :

1st : Filterable and invisible (virus), 2nd : Filterable and visible (" G " type culture of Hadley), 3rd : Unfilterable and visible (Bacterial), 4th : Spore form.

The morphological and biochemical behaviours of each cyclostage were studied as far as possible separately and are given below :—

1st : Filterable and invisible cyclostage (virus). This cyclostage gave no visible growth in agar or broth media, but on repeated transfers slowly passed into the second cyclostage of filterable but visible type of cultures similar to those described by Hadley.

2nd : Filterable and visible (" G " type culture of Hadley). These forms formed minute colonie on solid media and gave opalescent and hazy growth in broth media. This stage very often reverted to virus stage. The individual colonies almost invariably reverted but mass cultures steadily passed on to the next cyclostage of unfilterable forms. The organisms in this cyclostage were very minute and just resolvable with the highest power. Very fine granules were visible on staining with usual aniline dyes. In the impression films, obtained by touching the cover glass on to the colonies of the organisms, minute coccus forms predominated and very occasionally small rod-like forms could be differentiated. On staining such films with Zetnow's flagella stain a few minute rods with one long flagellum could be observed ; the number of such organisms, however, was not large. Fine granules mostly in clusters were preponderant. Most of the forms of this cyclostage were Gram negative.

The biochemical reactions were negative in almost all biochemical media, sugars were not fermented, nitrates were not acted upon, hydrogen sulphide, indol, and ammonia were not formed. Litmus milk turned slightly acidic without peptonization or coagulation. Gelatin was not liquefied, growth on potato was not visible and no diastatic action could be demonstrated. Pin-point colonies often developed after prolonged incubation but were too small to give definite information as regards colour and type of growth.

Spore formation was conspicuously absent, and aged cultures though viable failed to retain acid fast stain. Generally such cultures reverted to virus cyclostage and rarely into unfilterable cyclostage. Cultures could not be maintained in this stage for long and could only be re-isolated from the Chamberland candle filtrates of ordinary bacterial cyclostage.

3rd : Unfilterable and visible (Bacterial). Biochemical reactions of the bacteria which ultimately developed from the filterable stage gave negative reaction with almost all biochemical media. No acid or gas was formed in sugar media, nitrate was neither reduced nor formed. No ammonia developed in peptone. Indol and hydrogen sulphide were not formed. Litmus milk turned slightly acidic and peptonization slowly took place with clear yellowish upper layer, slightly reddish middle layer and bluish bottom layer. Gelatin was very slowly liquefied. The liquefaction was crateriform. The organisms were strictly ærobes, and gave a mealy growth with a dirty colour on potatoes. Diastatic action was absent.

The organisms were generally Gram negative but some times retained the Gram stain.

The size of the organism varied from 0·1 μ to 0·7 μ in thickness and 0·3 μ to 3·5 μ in length.

Flagella. The organisms were usually found to have a single polar flagellum but sometimes 3 to 5 flagella were observed occurring at both the polar ends. Peritrichous flagella were also noticed to occur and aged stock cultures occasionally developed this form. In some cultures non-motile forms without any flagella were obtained. This condition was usually rare and preceded the spore formation.

The organisms took acid and basic stains readily. The slime and capsules were absent.

4th : Spore stage. Very rarely spores were observed to occur and then the culture usually assumed a wrinkled appearance on the slant. The spores were oval and measured 0·3 to 0·5 μ in breadth and 0·8 to 1·2 μ in length. Experiments to find the conditions governing the spore formation did not give any results as all methods tried failed to stimulate spore formation. It appeared that under adverse circumstances the culture readily reverted to filterable and virus cyclostages.

Thus the organisms isolated differed in cultural and biochemical reactions from all known bacteria. Some resemblance to organisms isolated from the diseased tissues of tomato mosaic by Desai [1933] was observed but points of difference were distinct and many.

V. Serological reactions

(*a*) *Why carried out*

The repeated failure to induce the disease by the isolated organisms, their filterable and virus forms, suggested the investigation of the relationship of these to mosaic virus by serological tests.

These tests are all based on the principle that a serum containing antibodies produced against some organisms previously injected into the animal from which the serum is obtained, will give a positive reaction only with the organisms used in the injection or with some closely related type. Through the relative specificity of these reactions it is possible to study the relationships between different organisms and strains, and this method was utilized to study the inter-relationship between the bacteria, its filterable forms, and the mosaic virus. The tests were carried out by the method recommended by the Sub-Committee on serological methods of the Society of American Bacteriologists [1928].

The inoculations were made with cultures of bacteria, their filtrates, the virus (juice of mosaic leaves filtered through candle) and the juice of healthy leaves similarly treated.

(*b*) *Purity of antigens*

To obtain reproducible results it is highly essential that the material (the antigen) for injection into rabbits should be carefully purified and standardized. Each antigen was scrupulously tested for purity and guarded against chances of contamination. The method for obtaining each is given below :—

1. The virus antigen.—The young sugarcane leaves of Co. 213 showing heavy mottling were used throughout the experiment. The standard virus juice was prepared by crushing 50 grms. of leaves in 50 grms. of water and extracting 45 grams of the juice from the mixture through a cheese cloth. This juice was repeatedly filtered through the same filter paper till it gave clear brownish filtrate. The filtrate was then filtered through L_3 filter candle under 5 lbs. pressure into sterile tube. The glazed portion of the filter candle was wrapped in non-absorbent cotton wool, inserted into wide test tubes and the assemblage was sterilized by dry heat at 160°C. for 2 hours. The filtration was carried out according to the method of Martin. The candle was removed and the tube plugged with sterile cotton. The antigen was prepared fresh for each inoculation. The filtered juice remained clear and sterile.

2. Healthy juice antigen.—The material was prepared by the procedure employed for virus antigen from healthy sugarcane leaves of the same age as the mosaic ones.

3. Bacterial antigen.—The bacterial antigen was prepared by suspending the 48-hour growth on nutrient agar in sterile physiological saline. The growth was removed by platinum loops from the surface without disturbing the agar. The suspension was filtered through a sterile filter paper, standardized by opacity measurements and used immediately.

4. Filtrate antigen (for filterable forms).—The suspension of the bacteria prepared as mentioned above was filtered through sterile L_3 filter candle. The filter candle was assembled as previously described so that every possibility of chance contamination from air was avoided. The filtrate was prepared fresh each time and used immediately.

Scrupulous care was taken in handling various material used for the antigenic purpose and from beginning till the end kept under sterile condition.

(*c*) *Method of inoculation*

The antigens were injected into the marginal ear vein of the young rabbits by taking up the antigens in sterile Record syringes. A separate syringe was used for each antigen, and hands and everything used for inoculation were scrupulously sterilized before each inoculation. The amount of the dose was increased slowly

till the maximum was reached after five to eight inoculations. The experiments were repeated four times with different batches of rabbits at different times and the procedure employed remained the same throughout. The results obtained were similar. The detailed history of the immunization of one batch is given here for information, the others being omitted to save space :—

TABLE II

Date (1934)	I Mosaic leaf juice		II Bact. sus.		III Filtered Bact. sus.		IV Healthy leaf juice		V Bact. sus.		Remarks.
	Wt. lb. oz.	Dose c.c.	Wt. lb. oz.	Dose c.c.	Wt. lb. oz.	Dose c.c.	Wt. lb. oz.	Dose c.c.	Wt. lb. oz.	Dose c.c.	
19th Feb.	4–7		3–2		4–12		3–0		3–10		
20th ,,		2.0		0.2		2.0		2.0		0.4	
26th ,,	4–11		3–10		4–14		3–7		3–12		
27th ,,		2.6		0.4		2.6		2.6		0.6	
5th March	4–12		4–0		5–0		3–12		Died * 28th Feb. 1934.		
6th ,,		3.0		0.6		3.0		3.0			
12th ,,	4–11		4–2		5–2		3–14				
13th ,,		3.6		0.8		3.6		3.6			
19th ,,	4–12		4–3		4–12		4–2				
20th ,		4.0		0.9		†		4.0			
26th ,,	4–8		4–1		4–8		4–2				
27th ,,		4.0		1.0		3.8		4.5			
3rd April	4–10		4–3		4–3		4–8				
3rd ,,		4.5		1.2		4.0		4.5			
9th ,,	4–13		4–1		4–12		4–8				
10th ,,		4.5		1.5		4.5		4.5			
17th ,,	5–0		4–1		5–0		4–6				
20th ,,	Bled		Bled		Bled		Bled				

* Broncho-pneumonia and peritonitis—left lung badly affected, right lung partly. Serosanguineous fluid peritoneal and pleural cavities.

Swelling in ear : production of fibres in the vein due to irritation, consequent blocking of vein, evident only in the vein which received the inoculation.

† Drooping and bent towards the right side as if it were paralysed. Inoculation suspended.

(*d*) *Tests and their reactions*

Ten days after the last inoculation, the rabbits were bled from ear vein or by the heart puncture if more blood was required. The serum of each rabbit was

prepared separately and stored in an ice chest. The following reactions of the sera were studied :—

1. Agglutination of the organisms of sugarcane mosaic.
2. Precipitation of mosaic juice, filtrate of the organisms and healthy juice.
3. Hemolytic properties.
4. Complement fixation.

1. Agglutination.—The clumping or agglutination of bacteria under the influence of an immune serum is an important and well-known method for the differentiation of bacteria. The test is based upon the observation that the injection of bacteria into an animal causes the production of antibodies called agglutinins, which have the power of clumping (agglutinating) suspension of the same or closely related organisms when brought in contact with them under the proper conditions. Conversely, if one organism is agglutinated by different sera then the different organisms used for immunizing the rabbits have a very close relationship with the organism in question.

This test has been carried out very often and has always given similar results. The 48-hour old cultures were used for the test and these were suspended in physiological saline and filtered through filter paper. Light suspensions gave distinct and well defined end points. The sera were diluted in progression and mixed with equal quantity of the suspension in agglutination tubes. The tubes were kept at 56°C. for 30 minutes and in the ice chest overnight. The readings were taken next morning. Keeping longer at the high temperatures was not found to be useful.

Results of two typical experiments are given below :—

TABLE III

Agglutination reaction

Rabbits batch No. 2, bled on 19th December 1933 immunized by eight inoculations

Sera dilutions	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	1 : 2560	1 : 5120
Serum I virus	++++	++++	++++	+++	++	+	±	—
Serum II filtrate	++++	++++	++++	++++	++++	+++	+++	++
Serum III organism	++++	++++	++++	++++	++++	+++	+++	++
Serum IV healthy juice	—	—	—	—	—	—	—	—

Table IV

Agglutination reaction

Rabbits batch No. 3, bled on 20th April 1934, immunized by eight inoculations as given in Table II

Sera dilutions	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:2400	1:3200	1:6400
Serum I virus . .	++++	++++	++++	++++	++++	+++	++	+	—	—
Serum II filtrate . .	++++	++++	++++	++++	++++	++++	+++	++	—	—
Serum III bacterial .	++++	++++	++++	++++	++++	++++	+++	+	—	—
Serum IV healthy juice .	+++	++	++	++	—	—	—	—	—	—

The signs employed to record the results have usual serological significance as follows :—

++++ Complete agglutination *i.e.* sediment at the bottom of the tube with completely clear supernatant fluid.

+++ Complete agglutination. Sediment at the bottom and sides. Supernatant fluid quite clear.

++ Partial agglutination. Appreciable sedimentation at the bottom and sides. Supernatant fluid slightly turbid.

\+ Positive agglutination. Slight sediment at the bottom and sides. Supernatant fluid slightly clearer than the controls.

± Doubtful agglutination. Slight sediment on sides only and visible with lense.

— No agglutination. No sediment in the suspension. The suspension as turbid as the controls

2. Precipitation test.—This test was carried out to see if the virus and other antigens were precipitated by their homologous and heterologous antisera. For this purpose increasing dilutions of the sera were brought in contact with the antigens and the formation of visible precipitation noted. Each serum was put up against virus (mosaic leaf juice) filterable forms (filtrate of organisms) and healthy leaf juice and incubated in a water bath at 56°C. for 4 hours. The tubes were then transferred to an ice chest and kept overnight. Results were read following morning.

The result of a typical test is given below :—

TABLE V

Precipitation test, dated 7th July 1934

Sera dilutions		1 : 20	1 : 50	1 : 100	1 : 500	1 : 1000
Serum I virus	V.	+	±	—	—	—
	F.	±	±	—	—	—
	HJ.	±	±	—	—	—
Serum II *filtrate*	V.	+	±	—	—	—
	F.	+	±	—	—	—
	HJ.	—	—	—	—	—
Serum III healthy juice	V.	±	±	—	—	—
	F.	—	—	—	—	—
	HJ.	+	±	—	—	—
Controls . .	V.	—				
	F.	—				
	HJ.	—				

It would be seen from these results that the reaction was not very satisfactory. Modification in procedure did not help in making the doubtful precipitation definite.

Taking only definite precipitation as indicative of the affinity, the relationship between virus and filterable form was brought out. Virus serum reacted only with its own antigen and doubtfully with filtrate and healthy juice. Filtrate serum reacted definitely positive with virus and filtrate antigens and definitely negative with healthy juice while healthy juice serum reacted with its own antigen and doubtfully with virus antigen. In the more recent work of this nature on tobacco mosaic virus by Birkland [1934] it has been found that plant protein in the juice used for immunization interferes in the clarity of the reaction.

The plant proteins present in the juices used for immunization were estimated by finding total nitrogen and arriving at the amount of proteins present in the juices approximately :—

	Mosaic	Healthy
	Per cent	Per cent
Amount of nitrogen	0·01	0·01
Amount of protein calculated	0·06	0·06

It would appear from these that the amount of plant proteins in this case was too small to interfere in the reaction ; again qualitative tests of protein (namely biuret, xanthoproteic, Adamkeivicz, and Millon) were very faint in the juices employed for immunization.

3. Hemolytic properties.—The hemolytic action of the various antigens—namely the virus, filterable forms, bacteria and healthy juice—was investigated. For this purpose 0·25 c.c. of each of the antigens was mixed with 0·25 c.c. of unsensitized sheep's red blood corpuscles and 0·5 c.c. of saline in quadruplicate tubes and kept at room temperature for 30 minutes and at temperature of 56°C. for another 30 minutes. The tubes were then removed and stored in ice box overnight, the reaction being observed the following morning. The results were negative, that is the R. B. Cs. were not hemolysed.

4. Complement fixation.—Complement fixation test is based upon the observation that the combination formed between an antigen and its specific antibody has the property of fixing complement during the reaction. On the basis of this general law complement fixation can be used to detect the union of an antigen with its homologous or specific antibody. The test for such fixation is performed by placing together antigen, antibody and complement in suitable proporation as determined by previous titration and subsequently testing for the presence of free complement. If complement is not fixed, it indicates that the antigen and the antibody do not have power to unite or in other words, the antigen and the antibody are not specifically related. On the other hand the fixation of the complement in the mixture indicates that the antigen and antibody have combined owing to their specific affinities.

The test is very delicate and specific but there are cases in which, though the union of the antibodies with the antigen does take place, the complement is not fixed in the reaction. Thus whereas the positive fixation is a very strong proof of the union of antibody with the antigen the non-fixation of complement does not as a rule preclude the possibility of the union between antibody and the antigen in pure culture studies.

The test is much more specific than the agglutination test, requires careful attention to details and the preparation of number of accurately standardized serological reagents.

The complement fixation test was carried out with both heat stable and heat labile antibodies and the results of these tests are given below :—

The tests were carried out by mixing :

·25 c.c. of diluted sera (1 : 10)

·25 c.c. of the antigen

·25 c.c. of the complement of appropriate strength in minimum hemolytic doses (M.H.D.).

The mixture was kept at room temperature for 30 minutes followed by a temperature of 56°C. for 30 minutes. At the end of this period 0·25 c.c. of standard sensitized red blood corpuscles (R. B. Cs.)—were added and the trays containing the tubes kept at 56°C. for 30 minutes. The trays were then withdrawn from the water bath and kept overnight in the ice chest. The readings were taken next day.

The symbols and abbreviations used have their usual serological significance.

TABLE VI (*a*)

Complement fixation test with the unheated sera

Strength of the complements	Virus serum I against				Filterable form serum II against				Bacterial serum III against				Healthy juice serum IV against			
	V.	F.	O.	HJ.	V.	F.	O.	HJ.	V.	F.	O.	HJ.	V.	F.	O.	HJ.
8 M. H. D.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6 M. H. D.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5 M. H. D.	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4 M. H. D.	+±	—	—	—	±	—	±	—	—	—	—	—	—	—	—	—
3 M. H. D.	+±	±	±	—	+	±	+	—	—	—	—	—	±	—	—	±
2 M. H. D.	++	±	±	±	+±	±	+±	—	++	—	+	—	±	—	±	±

TABLE VI (*b*)

Controls

	Serum controls				Antigen controls			
	I	II	III	IV	V.	F.	O.	HJ.
5 M. H. D.	—	—	—	—	—	—	—	—
4 M. H. D.	—	—	—	—	—	—	—	—
3 M. H. D.	—	—	—	—	—	—	—	—
2 M. H. D.	—	—	--	—	+±	—	—	±

TABLE VII (*a*)

Test carried out with the heated sera

Strength of the complement	Virus serum I against				Filterable form serum II against				Bacterial serum III against				Healthy juice serum IV against			
	V.	F.	O.	HJ.	V.	F.	O.	HJ.	V.	F.	O.	HJ.	V.	F.	O.	HJ.
10 M. H. D. .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8 M. H. D. .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6 M. H. D. .	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5 M. H. D. .	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4 M. H. D. .	±	—	—	—	±	—	+	—	—	—	—	—	—	—	—	—
3 M. H. D. .	+±	—	±	—	+	—	+	—	±	—	±	—	—	—	—	±
2 M. H. D. .	+±	±	+	±	+±	±	++	—	+±	+	+±	—	±	—	—	±

TABLE VII (*b*)

Controls

	Serum controls				Antigen controls			
	I	II	III	IV	V.	F.	O.	HJ.
5 M. H. D.	—	—	—	—	—	—	—	—
4 M. H. D.	—	—	—	—	—	—	—	—
3 M. H. D.	—	—	—	—	—	—	—	—
2 M. H. D.	—	—	—	—	+±	—	—	±

Interpretation of the results of the complement fixation test indicated that the mosaic leaf juice (that is virus) has a distinct antigenic property as compared to healthy leaf juice. Mosaic juice was partly anticomplementary while healthy leaf juice was very slightly anticomplementary. Complement fixation with virus antigen was greatest when it was put up against its own serum ; less with filterable form serum, and organisms serum ; and least (even less than control) with healthy juice serum.

Filterable forms antigen when put up with different sera failed to fix any complement.

Organisms antigen fixed complement slightly when put up with its own serum and that of filterable form, otherwise the reactions were negative.

Healthy juice did not fix any complement even when put up with its own serum, thus showing a lack of antigenic substance in the leaf juice. This indicated that the antigenic substance in the mosaic leaf juice was the virus.

Antibodies were heat-stable, that is they could be heated without inactivation upto the temperature of 56°C. for half an hour.

The complement fixation was not much but it was sufficient to show the relationship between the virus, filterable forms and the organisms and supported the agglutination tests which were more definite. The difference from the reactions of the healthy juice serum was very marked.

The tests were carried out at Haffkine Institute, Bombay, by kind permission of Col. S. S. Sokhey, Director, Haffkine Institute. Dr. Wagle, Assistant Director, Haffkine Institute, greatly helped me in carrying out the tests proper for which I am much indebted to him. The results of other similar tests are omitted to save space.

(*e*) *Effect of sera on infectivity of the mosaic leaf juice*

These experiments were carried out to see what action was induced by the various sera on the infectivity of the mosaic leaf juice. The leaf juice filtered through a cheese cloth was used for these experiments.

The standard mosaic leaf juice was mixed with one tenth of its volume of appropriate serum and the mixture well shaken and inoculated into test plants within five minutes. Controls were also inoculated with mosaic leaf juice similarly diluted with water.

Scrupulous care was taken in inoculating the plants. Bundles of entomological needles mounted on corks were carefully sterilized and all other articles used were also sterilized. The hands were washed with an antiseptic in between the inoculations. The mixtures were slightly pricked with the needles in base of the leaves.

The appearance of mosaic in the plants was noted. The results of one of these experiments are given below :—

TABLE VIII

Treatments	Pots	No. of inoculated plants	Date of inoculation	No. of plants showing mosaic			
				30th Sept. 1933	2nd May 1934	10th May 1934	21st May 1934
Control (mosaic leaf juice).	9	20	23rd April 1934	0	4	11	18
Mosaic leaf juice+ healthy juice serum.	10	16	,,	0	3	7	11
Mosaic leaf juice+ mosaic serum.	10	20	,,	0	0	0	0
Mosaic leaf juice+ filtrate serum	10	20	,,	0	0	0	0
Mosaic leaf juice+ bacterial serum.	10	18	,,	0	0	6	12

These experiments definitely showed that the mosaic juice was inactivated or neutralized by its own serum and further that such inactivation was also caused by filterable form serum. The healthy juice serum as well as bacterial serum had no action on the virus. Thus a definite proof of the similarity between the filterable forms of the bacteria and the virus was obtained, as the two above-mentioned antigens produced such antibodies that they united and neutralized the virus. That animal serum as such has no such effect was clearly shown by other sera being unable to neutralize the virus.

Further experiments were conducted with the experimental plants of this series.

1. Some of the non-mosaic plants from each of the series were ground up and used for inoculating a further batch of plants to find out whether the symptoms of the mosaic disease were masked in these plants and the plants had become carriers of mosaic with mosaic virus multiplying in them. It was found that the non-mosaic plants of all the series failed to infect the plants inoculated with their juices showing thereby that the masking of symptoms did not take place and that the plants were really mosaic free.

2. Two of the plants showing no mosaic were reinoculated after 45 days with the mosaic leaf juice to find out whether the plants were susceptible, or an immunity inherited or artificial had developed in the plants.

When the plants were finally examined it was found that the non-mosaic plants from the control series did not take infection even on reinoculation, showing a kind of inherent resistance, while the non-mosaic plants of the virus-serum mixture series took infection on reinoculation very readily, showing that though the plants were susceptible the virus was neutralized *in vitro* in mixtures used for first inoculations. Non-mosaic plants of the filterable form serum mixture series when reinoculated with the mosaic leaf juice failed to take the infection and it might be due to an acquired resistance. Further experiments are necessary to come to any definite conclusions in this respect as the plants tried were only two.

Further experiments were conducted to see if the sera conferred immunity to the plants if they were inoculated into them for this purpose. The sera diluted ten times with sterile saline solution were inoculated into a batch of plants and the

standard mosaic leaf juice was pricked into them after 10 days. The results of this experiment are given below :—

TABLE IX

Treatments	No. of pots	Date of inoculation	Date of M. L. J. inoculation	Mosaic appearance 27th May 1934
Virus serum	2	23rd April 1934		None
Virus serum followed by mosaic leaf juice after 10 days	8	,,	3rd May	4
Filterable serum	2	,,		None
Filterable serum followed by mosaic leaf juice after 10 days	8	,,	3rd May	1
Bacterial serum	2	,,		None
Bacterial serum followed by mosaic leaf juice after 10 days	8	,,	3rd May	1
Control mosaic leaf juice . .	3	,,	,,	1

These experiments showed that the sera were not able to confer immunity to the plants ; even the mosaic virus serum failed to show any prophylactic power. It is possible that the time allowed for the immunity to develop might be too short. Again the amount absorbed by the plant might be too little. These points await investigation before the question of immunity is finally settled.

CONCLUSIONS

A filter-passing cyclostage of bacteria isolated from mosaic-infected sugarcane leaves has been clearly demonstrated. Pleomorphic unstable bacteria which have only a very short cyclostage as bacteria but a long one as filter-passers is a new phenomenon which has been discovered.

The relation of this stable, filter-passing, invisible cyclostage to the sugarcane mosaic virus has been definitely brought home by the study of the serological reactions and a series of inoculation experiments in which the mosaic virus was neutralised by the anti-filterable forms serum ; and these experiments suggested that the mosaic virus was the stable cyclostage of a species of bacteria which have a very unstable existence in the bacterial stage. The bacterial stage was antigenically different from the other cyclostages and also from the mosaic virus.

The species of bacterium isolated differs from all known bacteria in its biochemical and morphological characters as would be expected from its cultural behaviours. The new conception of the etiological agent of the mosaic disease of sugarcane requires to be investigated by independent workers in this field, and with the idea of stimulating such research the experimental data here recorded are put forward in their present preliminary stage.

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STINKING ROT OF SUGARCANE

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I. Occurrence

In July 1933 many sugarcane plants of Co. 300 and 313 were found to be wilting at Mushari Sugarcane Research Station. Most of the plants affected were found to be attacked by top shoot borers, but these insects very rarely kill the plants outright and the affected plants wilted in such a short space of time that other causes were looked for. The affected plants wilted completely within a fortnight and a peculiar fermenting smell was noticeable in fields having only a few plants so affected. The onset was sudden and all the leaves dried up though the weather conditions were very humid and favourable for the plants. The plants were examined and it was found that the disintergration and fermentation of tissues of the stem were taking place very rapidly from the top towards the bottom. The whole stem had become a mass of soft stinking pulp, and was found to be teeming with bacteria. The colour of the pulp was light grey and the offensive smell was very marked. In plants examined at this stage there was no sound tissue from which sections could be cut, but in plants showing incipient traces of the disease, the tissues a long way below the " dead heart " were found to be full of bacteria, mostly in intercellular spaces where the tissues had not disintegrated and in intracellular spaces as well in portions where fermentation was advanced. The vascular bundles were discoloured though these tissues did not disintegrate as did the other tissues. In advanced cases the leaves and the other dead tissues formed a favourable culture ground for many saprophytes, and the weather conditions favoured this secondary decomposition with the result that the leaves were black with moulds and other fungi. Insect maggots were also found in the fermented pulp, which mixed with the rain water and flowed all over the plant disseminating the offensive odour and attracting flies and other insects to the decomposing mass. The disease appeared to spread to adjoining stools which were quite healthy and without any top shoot borers ; such plants submitted suddenly to the rot and wilted within a short time.

II. Methods of isolation and cultures obtained

The bacteria responsible for this disease were isolated by plating from various affected tissues. The plating from pulped tissues invariably showed numerous saprophytic bacteria and yeasts, together with moulds and other saprophytic fungi. So many types of organisms existed in such tissues that it was difficult to find which one was responsible for the onset of the disease. The tissues below the rotted stem when minced up and plated showed a variety of bacteria and even here the causative organisms were hard to identify. The tissues still further below the rotted area were then tried, and from these it was found possible to isolate two types of bacteria, one white and other bluish on nutrient agar. The organisms grew well in the standard nutrient media and were obtained in pure culture by repeated plating in these media. No special medium was found to be necessary. The medium used contained :—

3·0 grams marmite
5·0 grams peptone
1000 c.c. water.
15 grams of shredded agar added to solidify the medium.

III. Pathogenicity

Pure cultures of these two types of bacteria were tried to find out their pathogenicity and reproduction of the typical disease by inoculations. At first the organisms were introduced into young plants by pin pricks, the organism being smeared over the surface and pricked in. Nothing beyond local lesion developed, which ultimately dried up in the case of the bluish type and in the case of the white type nothing except the wounds due to pin pricks were observed. Next the organisms were introduced into the plant by taking a plug out of the stem by a small cork borer. A culture was introduced into the hole, the plug was replaced and the stem tied up with tissue paper. In this case two plants out of six inoculated by the bluish culture showed disintegration of the tissues though not to a very great extent. Plants inoculated with the white organism remained healthy. Thus a slight pathogenicity of the bluish culture was brought out, while the white type was found to be unable to set up the disintegration. The infection took place only under excessively moist conditions.

The organisms were then introduced in borer holes of the borer-infected canes and a few marked as controls. It was then found that the bluish culture rapidly infected the tissues and set up the typical fermentation. Control plants remained unaffected and so did the plants infected in the borer holes by the white type. On trying to re-isolate the organism from the artificially infected plants it was found that the same two types of organisms were present and were readily isolated. It was at this stage suspected that the white type of organism though not parasitic was helpful to blue type which was pathogenic in setting up vigorous disintegration of the tissues.

Ase ries of plants was therefore inoculated with white and blue types separately and together, and it was found that the mixed culture was markedly more efficient in setting up the fermentation and disintegration of the tissues.

The organisms were not strong parasites and when introduced by small surface wounds did not infect the plants ; deep wounds and presence of dead tissues near about helped the infection and virulence developed after mass development of the organisms at the point of infection. These conditions were only fulfilled in the field when the organisms got entrance through borer holes or when mass culture of the organisms were introduced into healthy plants by contacts with the disintegrated tissues of neighbouring affected plants. The disintegrated tissues from decomposed lesions were found to be highly infective. The pathogenicity of both the organisms was also tested individually and collectively on hosts other than sugarcane. This was done because the bluish organisms resembled in some respects *Bact. marginale*, *B. aptatum* and *B. xanthochlorum* which are known to be pathogenic to solanaceous and other plants. Isolation of a similar organism from rotting potatoes has been reported by Hutchinson and Joshi [1915].

Potato, tomato, capsicum, brinjal and tobacco plants were infected by various methods and in varying conditions, but the organisms did not attack any of these plants. Bean plants were also inoculated but here too the organisms remained innocuous. Maize and *Euchlæna mexicana* were next inoculated but except for sugarcane the organisms were found to be non-pathogenic. The organisms were more infective in Co. 300 and Co. 313 than in Co. 213, though some amount of infection took place in other varieties of sugarcane tried. Later in the season, when the weather condition had become unfavourable, the infection did not spread rapidly unless the plant was covered up with a bell jar and kept in a warm place. Under these conditions the infection took its usual course.

Long cultivation on agar seemed to reduce the virulence of organisms for sugarcane.

When the artificial conditions of the test plants coincided with the natural condition of the bored plants the infection spread very rapidly and the typical smell and decomposition of the tissues took place.

IV. Biochemical reactions

Morphological observation and biochemical behaviour of the causative organisms are described below :—

These organisms were small rods $0 \cdot 6\text{-}1 \cdot 2\ \mu \times 1 \cdot 2\text{-}2 \cdot 2\ \mu$ in size without any spores or capsules, and were motile by single polar flagellum, which stained easily with any flagella stains.

Organisms were Gram negative and were easily discoloured by acid.

Ordinary Agar.—pH 6·5 at 30°C.

Colonies :—Greyish blue. Slight diffusion of greenish pigment in the agar surrounding the colony. Moist. Slightly raised.

Streak :—Greenish white, copious growth in water of condensation, diffusion of fluorescent pigment in the agar.

Gelatin :—Liquified within 48 hours. Crateriform, and fluorescent.

Nutrient broth.—Greenish yellow fluorescent turning deep green on shaking and assuming again the yellowish fluorescent appearance on standing. Clouding, slight ; pellicle at the top, no precipitate. The pigment decolourised by acid. Reaction of the medium neutral. Turning alkaline with evolution of ammonia.

Sugars, etc.—Glucose, saccharose, lactose, and glycerine fermented without gas. The reaction remains neutral. No fluorescence.

Milk.—Peptonized but not coagulated with zone formation. Top, bluish yellow, middle reddish blue and bottom unchanged in case of litmus milk. Production of yellowish pigment in ordinary milk with clear peptonization and slight precipitate at the bottom.

Potato.—Greenish yellow growth. Diffusion of greenish pigment in the potato tissues. Diastase present.

Uschinsky's solution.—Medium cloudy, slight pellicle.

Nitrate broth.—Nitrate not reduced to nitrite.

Indol.—Not produced. Hydrogen sulphide not formed.

Pectinase.—Present, dissolution of the middle lamella very marked. This property is responsible in the formation of pulpy mass in the rotting sugarcane as observed in the fields.

The characteristics of these organisms were compared with *B. aptatum*, *B. marginale* and *B. xanthochlorum* and the points of resemblance and difference are given in Table I.

TABLE I

Stinking rot	*B. apatatum*	*B. marginale*	*B. xanthochlorum*
1. Only one polar flagellum	Bipolar flagella	One to three polar flagella	One to three flagella
2. No spores	No spores	No spores	No spores
3. No capsules	No capsules	Capsules	?
4. Aerobe	Aerobe	Aerobe	Facultative aerobe
5. Gram negative	Gram negative	Gram negative	Gram negative
6. Not acid fast	Not acid fast	Not acid fast	Not acid fast
7. Agar colonies. Bluish, thin, smooth entire, flourescent, agar yellowish green, fluorescent.	Agar colonies smooth, flat, glistening, entire, internal fish scale markings. Agar green fluorescent.	Agar colonies cream becoming yellow, round smooth, thin, agar brilliant green.	Agar colonies yellow white to fluorescent
8. Milk peptonised but not coagulated, with zone formation. Top blue, middle reddish blue and bottom unchanged	Milk coagulated	Milk coagulated and turned green.	Coagulated and peptonised with green fluorescence
9. Gel. liquefied	Gel. liquefied	Gel. slowly liquefied	Gel. liquefied
10 Growth in sugars. No gas, neutral.	?	No gas, acidic. Dextrose	No gas, acidic. Dextrose and sucrose.
11. Ammonia produced	Ammonia produced	Ammonia produced	?
12. Nitrate not reduced	Nitrate not reduced	Nitrate reduced	Nitrate reduced
13. Diastatic action present.	No diastatic action	Diastatic action feeble	Diastatic action present
14. Fermi's sol:—Slight growth. Thin pellicle.	Thick pellicle and growth.	Heavy clouding and thick pellicle.	Some clouding
15. Conn's no growth	No growth	.....	.....
16. Indol not produced	Indol produced	No indol	Indol produced
17. Host (natural)	Host (natural	Host (natural)	Hosts
Sacharum officinarum	*Beta vulgaris*	*Lactuca sativa*	*Campanula ranunculus*
Non-pathogenic to *Capsicum annuum.*	*Tropaeolum majus Host (Artificial).*	.....	*Daucus carota*
Phaseolus vulgari, Solanum tuberosum.	*Capsicum annuum*	.....	*Lupinus nanus douglasi*
Nicotiana tabacum	*Lactuca sativa*	.....	*Nicotiana tabacum*
Lactuca sativa	*Phaseolus vulgaris*	.....	*Physalis alkekengi*
Zea Maize	*Solanum melongena*	.....	*Solanum tuberosum*
Euchlaena mexicana	.....	.....	*Vicia faba*

From this table it would be apparent that the organisms of sugarcane stinking rot belong to the group of *B. pyocyaneus* with a specialized parasitism developed against sugarcane and the name of *B. pyocyaneus saccharum* is suggested for the organism.

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PARASITISM OF *SCLEROTIUM ORYZAE* CATT.

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I. Introduction

Investigations on *Sclerotium oryzae* Catt. associated with the rice plants (*Oryza sativa* L.) were started at Pusa in 1911-1912 followin upon the discovery of plants affected by this fungus in Bengal and Burma. Since those preliminary studies were conducted, the disease which this fungus was supposed to be causing did not attain the serious proportions which were first apprehended and interest in it seems to have waned in consequence. In 1930-1931 however a serious disease of rice which appeared in the Rajasahi and Dacca divisions of Bengal was associated with this fungus and investigations were therefore re-s arted in October 1931 to determine the factors which made this disease a severe epiphytotic and to find suitable measures for preventing and controlling the disease. It was also intended that a watch should be kept for types of rice plants that would resist the disease.

II. Review of literature

The sclerotial disease of rice was first described by Cattaneo [1879] from Italy where it was found to do a good deal of damage to the rice crop. He found that the culm of the affected plants was covered with a black discolouration near the soil level which later spread to all the leaf sheaths. Due to the pressure exerted by the parasite within the tissues, the epidermis of the plant burst and the plants withered and died. The name by which we know this fungus was given by that investigator.

At the time of distributing an exsiccatum of *S. oryzae*, Briosi and Cavara [1886] published a short description of the disease and the causal organism. The symptoms described by them laid stress on the black discolouration of the culm near the soil level and its ultimate rotting and they confirmed Cattaneo's observations in other respects.

The earliest mention of *S. oryzae* in India is by Butler [1913] who found it in association with the rice plants in Upper Burma. Some of the partially sterile plants did not have any insect attack but they were infested by the sclerotia of the fungus. In addition to this sterility, the plants showed the symptom of excessive tillering or the development of green sterile shoots from the basal nodes of the stock. The base of such plants was discoloured and was also invaded by fungal hyphae. The specimens were brought to Pusa where the associated fungus was identified as *S. oryzae*.

Very shortly afterwards Shaw [1913] published the results of an investigation carried out by him on this fungus. The chief symptom of the disease was stated by him to be tillering. The culm gradually turned yellow and died and if any panicle was borne, it was poorly filled and the grain was extremely light. The details of the cultural study conducted by him considerably supplemented the rather meagre description of the fungus given by Cattaneo [1879]. No infection experiments in pots or n the fields seem to have been conducted but the pathogenicity tests carried on in potato (Roux) tubes in which seedlings were grown and infected gave 70 to 80 per cent success provided the mycelium used for infecting the plants was young and vigorous.

A stem rot of rice ascribed to this fungus was described by Tisdale [1921] from Louisiana in the U. S. A. The affected plants had more or less entirely collapsed and the panicles were partially sterile. The stem nearer the ground, two or three node from the base, appeared darker and showed sclerotial infestation. The internode where the irrigation water stood was found to be completely destroyed, the epidermis alone remaining intact. Infection experiments conducted by him with the fungus were hundred per cent successful.

In Indo-China Vincens [1923] considered a sclerotial fungus to be the cause of the ' tiem ' disease of rice. The disease was characterised by a sparse growth of the plant, the stems of which became thin and leaves small, rigid, and pale.

He isolated from the base of dried leaves and from within the leaf sheaths a fungus which resembled *S. oryzae* in all respects.

According to Reyes [1929] the sclerotial disease of rice appeared to be the most destructive in the Philippines. The disease was detected in the field when the leaves began to turn yellow indiscriminately, followed by a stunting of the plants. Young leaves of the diseased plants were somewhat darker green and they tended to become stiff and erect. Three *Sclerotia* were isolated which were identified as three strains of *Sclerotium oryzae* and were designated as A, B, and C strains. Infection experiments in pots with the three strains gave 100 per cent success with a susceptible type of rice. Field tests gave from 20 to 93 per cent success.

According to Park and Bertus [1932] the sclerotial disease seemed to have been doing a good deal of damage to the rice crop in Ceylon. They state that it had been noted by Petch even as early as 1913. Park and Bertus found that the disease developed in a nursery in the seedling stage and one variety was so badly damaged that only a few of the plants survived. Examination of the affected plants invariably showed the presence of sclerotia which were identified as those of *S. oryzae*. Plants approaching maturity showed an unusual production of young shoots or tillers and the stalk gradually turned yellow and died. The infection experiments with seedlings gave on the whole successful results. Abnormal tillers were also noticed when infections were made on advanced plants, some affected plants even succumbed to the disease.

Symptoms of sclerotial disease as it occurs on the rice crop in Arkansas, Texas and Louisiana in the U. S. A., are described by Tullis and Cralley [1933]. The earliest manifestation of the disease was the presence of small black lesions on the outer leaf sheaths of the plants at the water line. The stem at this region showed considerable rotting as the spots enlarged and finally the tillers that were normally produced at the base were killed and the whole plants later succumbed to the disease.

From the rather detailed review of the symptoms of the disease of rice supposed to be caused by *Sclerotium oryzae* given above, it will be apparent that (1) the seedling disease due to this fungus, under natural conditions, has been recorded only in Ceylon, (2) that the phenomenon of excessive tillering has been associated with the disease by investigators at Pusa and Ceylon, and such a symptom has not been observed in Italy, Indo-China, the Philippines or the U. S. A., (3) that in these countries the most important symptom of the sclerotial disease is a stem-rot which leads to a yellowing and drooping of the plants and finally to their death and that these symptoms have not been recorded in India. In all cases however the sclerotia of *S. oryzae* were unmistakably present in the culms. There are thus considerable differences in the symptom-picture of the

disease as described, for in India the acute rot of the stem has not been observed either by previous investigators or by the writer while the symptoms noted here have not been found elsewhere.

III. *Sclerotium* spp. included in the investigations

Cultures of *Sclerotium oryzae* and other *Sclerotium* spp. for the study were assembled from several sources. In many instances straw bearing the sclerotia was secured from different geographic locations in India from which the fungus was isolated. Many of the cultures were kindly sent by pathologists in other countries.

In making the isolations, pieces of straw bearing the sclerotia were washed with mercuric bichloride solution (1 : 1000) and then with sterile water. These pieces were transferred to a sterile Petri dish where the sclerotia were aseptically teased out and transferred to oat agar (five per cent Quaker oats and one and one half per cent Bacto agar). Pure cultures of *S. oryzae* and other *Sclerotium* spp. were thus obtained.

As soon as the cultures were isolated or were received from abroad, single sclerotia were picked on a transfer needle and planted on potato-glucose agar (one litre of clear infusion from 200 grams of peeled and cut potatoes + 2 per cent glucose + 2 per cent Bacto agar). The cultures of *S. oryzae* produced after about 10 days a coral pink colour (Ridgway : ' Colour Standards and Nomenclature ') on this culture medium, which later became jasper red. This characteristic helped in sorting out the cultures of *S. oryzae* for none of the other sclerotia-forming fungi that were encountered in this study had this property. Production of various shades of pink by this fungus has been noted by Shaw [1913] and Park and Bertus [1932] on some substrates including maize-meal agar. When that agar was made according to the formula of Shear and Wood the cultures did not develop the pink colour in the writer's studies. The colour on the contrary was olivaceous to greenish mixed with slaty grey. The colour production was noted when the cultures of *S. oryzae* were grown on the following other media : Lima bean agar, Brown's synthetic agar and oat agar. Deepest colour production was however on the potato-glucose agar. It was noted that the intensity of the colour produced depended on the variety of potato used in making the medium and on the strain of *S. oryzae*. For example the intensity of colour produced on a medium made from potatoes sold at Pusa as ' Hill potatoes ' was not so intense as on that made from local (' Dehati ') potatoes. Likewise isolate Nos. 1, 3 and 4, (Table I) produced more intense colour than the rest of the strains.

When the fungus was grown in flasks on potato-glucose agar, the progressive change in the colour of the medium could be watched. Some isolates changed the colour quickly while the others were rather slow in doing so. Colour production did not seem to depend on light for inoculated flasks placed in a dark

chamber developed the colour. If the flasks were left for eight to ten weeks, the colour slowly faded and after sometime a faint indication of pink could be seen. When the isolates were grown on potato-glucose broth (the above ingredients without the agar) in flasks, colour was produced with equal intensity and when the liquid was filtered, the filtrates were scarlet. The description of the cultures included in the study follows :—

TABLE I

Cultures of Sclerotium *spp., used in the comparative study*

Serial No.	Description
1	Isolated from straw obtained from Dacca. October 1931. *S. oryzae.*
2	Sent by M. Park, Ceylon, February 1932. Chromogenic strain of *S. oryzae.*
3	Sent by E. C. Tullis, U. S. A. Originated in a single ascospore from *Leptosphaeria salvinii* Catt. April 1932. *S. oryzae.*
4	Sent by U. Thet Su, Burma. May 1932. *S. oryzae.*
5	Sent by H. Chowdhury, Punjab, October 1932. *S. oryzae.*
6	Sent by T. Nakata as *S. oryzae.* September 1932.
7	Isolated from straw, Anakapalle. *S. oryzae.*
8	Isolated from straw from Mymensingh. *Sclerotium* sp. October 1931.
9	Sent by M. Park, Ceylon (? *S. oryzae,* non-chromogenic strain*).
10	Sent by U. Thet Su, Burma as *S. oryzae,* large size.
11	Sent by T. Nakata, Japan, as *S. sphaeroides.* May 1932.
12	Sent by T. Hemmi, Japan, as *S. oryzae sativae* Sawada, May 1932.

(*See appendix)

In addition to the above, *S. oryzae* has been isolated from rice straw received from Jorhat in Assam, Kalashakaku and Karnal in the Punjab, Karjat in the Bombay Presidency and from Kashmir. *Sclerotium* resembling accession No. 8 has also been isolated from rice straw from Kalashakaku, Karnal, Chinsurah, Motihari and Maruteru. Two more sclerotia-forming fungi were isolated from

some of the rice straw, one of which resembled a culture sent by Dr. T. Nakata under the name *Sclerotium microsphaeroides* Nakata, while the other one is of the *Rhizoctonia* type. Studies with these are not yet complete and they have not been included in this report. None of the Indian isolations resembled the fungus sent by Dr. T. Hemmi as *Sclerotium oryzae-sativae* Sawada and as it did not seem to occur here, the culture and the sub-culture were killed in order not to introduce a new fungous parasite into the country.

IV. Morphological studies

Extensive experiments to determine the influence of physico-chemical factors on *S. oryzae* were planned but when the pathogenicity tests of 1932 indicated that the fungus under Pusa conditions did not produce disease, laboratory work was considerably slackened and it was stopped completely when the results of 1933 experiments confirmed the previous year's negative results. Enough however was done to determine a few growth characteristics and morphological features so that it is possible to define the *Sclerotium* spp., associated with the rice straw in India.

S. oryzae Catt.—This *Sclerotium* seemed to have a preference for those media which contained an infusion from cereals or vegetables. On Brown's synthetic agar, which contains starch, growth was fair but on completely synthetic media like the Czapėk's, Richards' and Coons' growth was extremely poor and sclerotial formation scanty. Production of a coral pink to jasper red colour by this fungus on certain substrates has already been noticed.

A white fluffy growth began to appear on potato-glucose agar the third day after making a transfer and sclerotia appeared on the ninth day together with smoky lobed appressoria. The mycelium became olivaceous to black at the edge of the medium in the test tube but not in all the strains, especially isolate No. 3. Some isolates (*viz.* 3 and 7) formed more aerial than submerged mycelium, which was of a pale drab gray to smoky gray colour. A thick layer of sclerotia was formed just beneath this aerial growth.

The young mycelium from a potato-glucose agar tube was hyaline but as it aged it developed a pale amaranth pink colour. Branching was profuse and at the base of the branch there was a slight constriction and a basal septum and this faintly resembled the mode of branching in *Rhizoctonia solani* Kuhn. The older mycelium was highly vacuolated and chlamydospore-like structures mentioned by Shaw [1913] abounded. It was observed that the mycelium from onion agar where the substratum was not discoloured, except at the edges, where it turned black, was sub-hyaline. The mycelium was from 3 to 5 μ broad and the length of the cells varied from 40 to 225 μ.

The cultures were grown on oat agar for obtaining an abundance of sclerotia for the purpose of measuring their diameters. The cultures were a month (30 days) old at the time of measurement. The sclerotia were picked from any random

place, mounted in lacto-phenol (one part each of lactic acid, phenol, glycerine and water by weight) mounting medium and two diameters at right angles to each other were measured and were then averaged. By accident 210 sclerotia were measured at the first instance and the same number was measured in the other cases likewise for the sake of uniformity. There is thus not any special virtue about that number.

The frequency distributions of the sclerotial measurements, the range, the mean and its standard error and the coefficient of variability are given in Table II.

Table II

Frequency distributions and biometric constants in microns of diameters of seven isolates of Sclerotium oryzae

Isolate No.	Classes																	Biometric constants.	
	156	182	208	234	261	287	313	339	365	391	417	443	469	495	522	548	574	600	*Mean	*Coefficient of variability per cent
1	...	5	14	56	57	46	27	10	4	1	...	...	...	...	...	...	...	...	266±2·63	14·3±0 71
2	...	...	11	34	47	47	43	11	7	3	4	3	1	...	...	...	...	...	286±3·32	16 8±0 84
3	3	17	31	41	69	33	15	1	...	...	...	...	...	...	...	...	...	...	242±2·48	14·5±0·72
4	...	4	35	61	79	24	6	...	1	...	...	...	...	...	...	...	...	...	284±1·88	10·9±0·54
5	...	...	2	7	7	8	18	13	25	20	29	20	21	13	8	10	8	1	405±5·97	21·3±1·08
6	...	...	...	2	9	20	37	40	50	36	12	4	...	...	...	...	...	...	347±2·46	10·3±0 51
7	...	...	6	17	29	36	46	34	19	14	8	1	...	...	...	...	...	...	310±3·43	16·0±0·80

* In this and other tables, standard errors are always given.

In the data recorded in Table II, it will be noted that the range of the diameters of the sclerotia of the different cultures varied from 156 to 600 μ and the mean diameters from 242 to 405 μ. The isolate with the largest mean diameter showed rather high variability in size so that it would have been better had a larger number been measured. The measurements given are in general accordance with those recorded by Shaw [1913] and Tisdale [1921]. The mean diameter of the Ceylon strain is 286 ± 3·32 μ and the diameter of this same *Sclerotium* according to Park and Bertus [1932] is 374·5 + 4·3 μ. This discrepancy can be explained by the fact that they did not secure their sclerotia at random but selected them from one half inch from the point of inoculation in a maize agar tube. It is possible therefore that this is the cause of the discrepancy.

Briosi and Cavara [1886] found the sclerotial diameters to be 150-180 μ but Ashby states (in a personal communication) that he measured 16 sclerotia from their exsiccatum which gave a range of 225-330 μ with a mean diameter

of 270 μ. The writer measured ten sclerotia from their exsiccatum that is in the Pusa herbarium and the range obtained was 208-339 μ with a mean diameter of 262 μ.

It was of interest to determine whether the diameters of sclerotia of culture No. 3 (sent by Dr. Tullis) which had its origin in a single ascospore (in its *Leptosphaeria* stage) showed any consistency in size in view of the variation shown by the sclerotial bodies. The fungus was grown on oat agar for thirty days, a sub-culture for the second clonal generation made, and then two hundred and ten sclerotia were measured. The second clonal generation was also grown for thirty days, a sub-culture for the third clonal generation made, and the sclerotia were measured. The third generation sclerotia were also measured after thirty days. The frequency distributions and other biometric constants will be found in Table III.

TABLE III

Frequency distributions of sclerotial diameters of three clonal generations

Progeny	Classes in microns								Biometric constants	
	156	182	208	234	261	287	313	339	Mean ± S. E.	C. V. ± S.E.
3_1	3	17	31	41	69	33	15	1	242 ± 2·48	14.5 ± 0·72
3_2	1	16	23	39	76	26	17	12	258 ± 2·57	14.4 ± 0·71
3_3	2	13	30	47	66	36	14	2	251 ± 2·43	14.0 ± 0·69

These frequency distributions were then compared using the Pearsonian method suggested by the writer [Mundkur, 1934] cell by cell with a view to see whether they could be considered as having descended from the same parent. The value of χ^2 and P (Fisher's table) are given in Table IV.

TABLE IV

Comparison of sclerotial diameters of three clonal generations of S. oryzae, *culture No. 3*

Generations compared	χ^2	P(between)
3_1 and 3_2	11.8645	0.10 to 0.20
3_1 and 3_3	1.6082	0.98 to 0.95
3_2 and 3_3	12.0625	0.10 to 0.20

From the data recorded in Table IV it will be observed that the values of P are sufficiently high. According to Fisher [1932] if P is between 0·1 to 0·9 there is no reason to suspect that the differences in mean diameters are such as to rule out the hypothesis that the sclerotia belong to the same general population or a common parent. It is only when the value of P is 0·02 or below that a strong indication points to their not belonging to such a population. As all the values of P in the table are within the non-significant range, it can be concluded that the mean differences in the diameters of the sclerotia are not such as to preclude the possibility of their belonging to the same parent which we know they do in terms of the experiment.

Tullis [1933] has reported that *Helminthosporium sigmoideum* Cavara is the conidial stage and *Leptosphaeria salvinii* Catt., the ascigerous stage of *S. oryzae*. He obtained the former stage in culture media as well as on affected plants while the latter stage (ascigerous) was obtained only in the affected leaf sheaths and culms. This conidial stage was also obtained by him in cultures of *S. oryzae* sent by the writer from Pusa. No such conidial stage was observed in culture media at Pusa but when the agar from the culture tubes bearing the mycelium was asceptically taken, placed on a sterile watch glass and incubated in a sterile moist chamber in diffuse light, conidial production has been obtained. The Indian and Burmese strains of the fungus belong therefore to *H. sigmoideum* in their conidial stage and therefore to *L. salvinii*, the ascomycetous stage, by analogy.

Sclerotium sp.—Isolates included under this heading comprised accession Nos. 8, 9, 10 and 11. Park and Bertus [1929]* considered this as the non-chromogenic strain of *S. oryzae* but their detailed account which seems to have been sent up for publication* (*vide Adm. Report, Ceylon Dept. of Agric.* 1931, p. D 111) is not however yet available.

The culture sent by Dr. Tanaka was labelled '*S. sphaeroides* Nakata, formerly called Sakurai's *Sclerotium* No. 2.' A search in literature to find out where the fungus may have been described by him proved fruitless, neither could it be ascertained from the author himself.

Cultural studies revealed that these four isolates belonged to one fungous species which differed from *S. oryzae* in several respects. Grown on oat or potato-glucose agar media, there was not any production of colour in the substrates which retained their original tint. The fungus did not seem to have any discrimination against synthetic media for it grew well on all the common media, vegetable, cereal or synthetic. It was noted that sclerotial formation started within six days of planting a sclerotium of the fungus on a nutrient medium. After some time aerial growth appeared to be rather scanty, for all the aerial hyphae had by this time been turned into sclerotia of which there was an abundance. Hyphae within the substratum were however plentiful.

* See appendix.

The young mycelium from a potato-glucose agar tube did not show much branching but the older mycelium was profusely branched. It was hyaline and there was not any development of colour within the hyphae, however old. Chlamydospore-like structures and the appressoria were absent. The constriction at the base of a branch was more of the *Rhizoctonia* type. Septation was close, the length of cells being 25 to 80 μ and the breadth from 3 to 5·5 μ. Clamp connections were rather rare.

The sclerotia appeared smooth but they really had a rough surface due to slight protrusions. The sclerotia were spherical, sub-spherical or oval, and sometimes three to four sclerotia were fused. To begin with they were whitish but later they turned brown and finally to fuscous black. Hardly any of them were submerged within the substratum. Their diameters ranged from 217 to 713 μ. Two hundred and ten sclerotia of each culture were measured and their mean diameters and other biometric constants are recorded in Table V.

TABLE V

Biometric constants of sclerotia of Sclerotium sp.

Isolate No.	Range	Mean ± S. E.	Coefficient of variability ± S.E.
	μ	μ	Per cent
8	269 — 713	443 ± 5.16	16.8 ± 0.84
9	269 — 713	465 ± 5.95	18.6 ± 0.93
10	269 — 713	446 ± 4.92	15.9 ± 0.79
11	217 — 609	442 ± 5.93	19.4 ± 0.98

The data recorded in Table V shew that there is not much variation in the mean diameters of the sclerotia, excepting that the culture from Ceylon seemed to have rather large sclerotia.

The question of nomenclature of these isolates now remains to be settled. This fungus (all the four isolates) differs from *S. oryzae* in the inability to produce pink colour on certain substrates, in the ability to grow profusely on synthetic media, in the mycelium being hyaline, in the absence of smoky lobed appressoria and the chlamydospore-like structures, and in the size of sclerotia which are decidedly larger. It has not formed the *H. sigmoideum* stage even though several methods to induce the stage were tried. It can by no means be considered therefore as the non-ehromogenic stage of *S. oryzae.*

Nakata also seems inclined to consider this as a distinct fungus for he calls it *S. sphaeroides* but it cannot be designated by that name as this binomial has already been used by Massa [1912] to describe a sclerotial fungus on *Lychnis dioica* L. in Italy.

But the branching of the hyphae in this fungus is very typical of *Rhizoctonia*, for there is the typical constriction at the base of the branch and also the basal septum. The sclerotial surface is not smooth but rough as in the case of *Rhizoctonia* and the fungus has therefore to be placed in that genus. Ashby to whom a culture was sent for the purpose of identification compared it with a culture of *R. microsclerotia* deposited at the Centraalbureau voor Schimmelcultures, Baarn, Holland by Dr. T. Matsumoto and found that it differed from it in several respects. But Ashby states (in a personal communication) that the culture sent from here tallied in several major respects with the published description of *R. microsclerotia* given by Matz [1917, 1921]. Even though Matz mentions a corticial stage which has not been observed in the culture here, the writer is disposed to agree with Ashby. As an authentic culture of Matz's strain is not apparently available, cultures were submitted to Dr. J. Matz for his judgement in the matter and he wrote :

> "Two of the tubes contained unmistakably the fungus *Rhizoctonia microsclerotia* and were inscribed with the number 59. I am sorry I am unable to send you cultures of my *Rhizoctonia microsclerotia* or *R. alba*."

(The number 59 mentioned above refers to number 8 used in this publication).

The Indian, the Burmese, the Ceylonese and the Japanese cultures are therefore identified as those of *Rhizoctonia microsclerotia*. A culture of a *Sclerotium* isolated from the Philippines rice straw was received from Dr. C. J. Humphrey, and it was identical with the cultures of *R. microsclerotia*. A culture of isolation No. 8 has been deposited at the Centraalbureau at Baarn as it has been authenticated by Dr. Matz, Mr. Ashby and the writer.

V. Infection experiments

Infection experiments were started in the rice season of 1932 both in pots and fields. The pots were usually of glazed earthenware and were filled with soil from the rice fields. An adequate number of them were-steam-sterilised under pressure for two hours. Four plants were usually sown in each pot, unless otherwise stated.

There were three plots for conducting the field experiments, each 26 × 18 feet, lying side by side, in a north to south direction. In 1932 it was noted that these plots were badly infested by termites. The soil to a depth of a foot was therefore removed and replaced with soil from a good rice field in April 1933. The centre plot served as a check plot. The other plots and the pots were infested

with cultures of *S. oryzae* in the fourth week of April each year. These cultures were grown on large quantities of a sterilised mixture of paddy and straw in 1500-c.c. flasks. The fungus was allowed to grow in the flask for six weeks during which time it formed profuse sclerotia. Contents of six such flasks were added to each of the two plots which were ploughed soon after and irrigated. The pots were likewise infested with large quantities of the fungus and irrigated, and water was allowed to stagnate both in the pots and the fields. The plots were re-infested in 1933 and 1934 and during these years additional infestation was also done after the crop was eleven weeks old. In 1934 further infestation was done by adding straw bearing the sclerotia to the soil in the two infested plots.

At the time of sowing, the seed was mixed with the sclerotia in 1932 and 1933 but not in 1934. The plots were sown with seedlings raised in the nurseries. They were dipped at the time of sowing in a suspension of sclerotia. It will therefore be noted what great care had been taken to see that infestation of the soil by the fungus was particularly heavy. The infectivity of the soil was therefore all that could be desired provided of course that the fungus was pathogenic.

Experiments in 1932.—The seed for these experiments was obtained from the Dacca Agricultural Farm where the disease had appeared in an epidemic form in 1930-1931. The seed was designated *Indrasail* and was supposed to be particularly susceptible to the disease. There were 24 pots containing sterilised soil and the rest had ordinary soil. The results are tabulated in Table VI.

TABLE VI

Infection experiments in 1932 with cultures of Sclerotium oryzae

Date of sowing	No. of pots	Total plants	Culture number	Number attacked
June 10 . .	5	20	1	None
June 10 . .	4	16	check	None
June 10 . .	4	16	2	None
June 10 . .	5	20	3	None
June 10 . .	6	24	4	None
June 28 . .	4	16	1	None
June 28 . .	6	24	3	None
July 19 . .	5	20	1	None
September 21 .	6	24	1	None

The tests conducted in July and September were done in ordinary flower pots in unsterilised soil. None of the plants showed any symptoms of the disease at any time. There were not any lesions or any excessive tillering and the panicles were normally filled with grain. In the pots sown in June and July the fungus was not only incorporated with the soil and given with the water on two occasions (July 13th and October 10th) but agar bearing the mycelium was placed within the leaf sheaths. There were no symptoms of the disease even then. The pots sown in July and September flowered rather late, that is in November, but the experiment was terminated at the same time as the June pots and it could not therefore be ascertained whether they bore well-filled panicles or not. After harvest the straw was stored in a dry place and when examined a week later it showed the presence of a large number of sclerotia.

Field tests in 1932.—The seed was sown in beds on the 3rd of June 1932 and transplanted on the 16th of July 1932. The crop was carefully watched. In July and later, several plants suddenly dried up and died. These were picked and examined when it was found that they had all succumbed to termite attack. Such deaths took place even in the check plot. The crop suffered from leaf spots due to *Helminthosporium oryzae* Breda de Hann, but of the symptoms of the sclerotial disease, there were none. There was no excessive tillering or stem rot and the plants bore well filled panicles. After harvest in November the straw was examined for the sclerotia. Several of the plants bore these in the leaf sheaths but they became appreciably greater in numbers after about a week when the straw had partially dried.

Experiments in 1933.—The tests during this year were carried out in the same manner as in the previous year, except that the quantity of inoculum in the plots and pots was more than last year. Furthermore three more isolates collected during the previous year were also tested for their pathogenicity in the pot experiments. The seed for the experiments was secured from the Economic Botanist, Dacca and was designated Chinsurah No. 1 by him.

Pot experiments.—The results of the pot experiments are recorded in Table VII. The soil in the pots this year was not sterilised.

TABLE VII

Infection experiments in pots with Sclerotium spp. *in 1933*

Date of sowing	No. of pots	No. of plants	Culture used	Number attacked
3rd June 1933 . . .	5	20	1	None
3rd June 1933 . . .	6	24	3	None
3rd June 1933 . . .	6	24	4	None
3rd June 1933 . . .	6	24	5	None
7th June 1933 . . .	3	12	6	None
7th June 1933 . . .	6	24	7	None
7th June 1933 . . .	4	16	8	None
7th June 1933 . . .	5	20	11	None

As glazed pots were not available for all the tests, ordinary flower pots were used for isolates Nos. 6, 7, 8 and 11.

None of the plants showed any of the disease symptoms caused by *S. oryzae*. There was no abnormal tillering and the plants were not sterile. In ten ordinary pots seed had been sown but the soil was not infested with the fungus. These also remained healthy. It was noted that the plants did not bear well and this feature was manifest in the control pots also. On the whole the Dacca seed did not seem to be well adapted for growth under Pusa conditions.

Several trials were made in June-July 1933 in small four inch pots to see if the disease could be produced in the seedlings. There were twenty such pots and to each one of them large quantities of the fungus were added. Six seeds were sown in each pot and the seedlings were kept under observation for about five weeks, but there were no signs of any seedling disease. The pots were then abandoned.

As Shaw [1913] and other investigators obtained successful results when they infected the plants growing in test tubes, similar tests were done in the month of July, 1933. The technique followed was the same as described by the writer [Mundkur, 1926] and as a liquid culture medium Shive's R_5C_2 solution was used. In addition to large test tubes, one inch broad and nine inches tall, seedlings, were raised in Roux tubes. In all there were twenty-eight large test tubes and thirty-six Roux tubes. The seed used in these studies was the same Dacca seed as used in the pot experiments and it was, prior to use, surface-sterilised for three hours in a solution of calcium hypochlorite (bleaching powder) as recommended by Duggar

and Davis [1919]. Two seeds per test tube were placed on 6th July 1933 and these test tubes were kept in a warm place. The seed emerged within four days. On the tenth day when the seedlings were about two inches high, they were infected with sclerotia and vigorously growing mycelium from isolates, No. 1, 2, 3, 6, 7, 8, 9, 10 and 11. There were six tubes for each culture and ten served as controls.

The tubes were placed first in diffuse light and later they were transferred to strong light. On the 14th day, that is four days after infection, some of the plants showed lesions and this symptom became general two days later in all the infected tubes. The mycelium was also seen to be growing well. Three weeks after the sowing of the seed in the tubes, the plants began to die and most of them were dead within the twenty-fifth day. The plants in the controls showed no such symptoms and remained very healthy.

The infected tubes were abandoned and on the twenty-seventh day five of the control tubes were infected with isolate No. 1. The plants completely succumbed to the disease within twelve days. The rest of the controls remained healthy.

Field experiments in 1933.—These were done on the same lines as in the previous year except that more inoculum was added to the soil. The plots were sown with four week old seedlings on 12th July 1933 and the growth of the crop in all the three plots was good. The crop suffered from leaf spots due to *Helminthosporium oryzae* and to a slight extent, *Priricularia oryzae* Br. and Cav. The plots were harvested in November. There was no excessive tillering, sterility or any stem rot. The infected plots yielded 2 lbs. 6 ozs. and 2 lbs. 13 ozs. each and the check plot yielded 2 lbs. 9 ozs. seed. The yield on the whole was not good.

The leaf sheaths of the growing plants from the infected plots were examined from time to time to see if the fungus was present. In October some leaf sheaths from the infested plots showed the presence of the fungus within the tissues and the appressoria mentioned by Tullis and Cralley [1933] were noticed together with a few sclerotia. After the straw was harvested, a large number of sclerotia were noticed in the sheaths. No such fungous infestation was observed in the culms or leaf sheaths of plants in the check plot and, when the straw was again examined a month after harvest, there were no sclerotia.

Experiments in 1934.—The infection experiments were repeated on the same lines as in previous year with this exception that the seed sown in pots was of Type 31, a type obtained from the Botanical Section at this Institute. Instead of seed, seedlings were sown in the pots, soil in which was not, however, sterilised. The isolates used for infestation of the soil in the pots were No. 1, 3 and 7. Seedlings of Shoemed rice, which is a very susceptible type in Arkansas and Louisiana to the stem-rot disease and which had been kindly sent by Dr. E. C. Tullis, were transplanted in the plots on July 3, 1934.

Pot experiments in 1934.—The results of the pot test are given in Table VIII.

TABLE VIII

Infection experiments in pots with Sclerotium oryzae

Date of sowing	No. of pots	No. of plants	Culture used	Number diseased
6th July 1934 . . .	16	64	No. 1.	*Nil*
6th July 1934 . . .	14	56	No. 3	*Nil*
6th July 1934 . . .	10	40	No. 7	*Nil*
6th July 1934 . . .	12	48	control	*Nil*

It will be noted that as in previous years, no disease was recorded in the pots.

Field experiments in 1934.—On the 18th of August, additional quantities of sclerotia and mycelium growing in giant cultures were added to the soil in the two plots. In September and October, plants were up-rooted and examined for sclerotia but none were found to be infested. Several plants in the infected and the check plot died in October due to termite attack. The straw was again and again examined with negative result. In late October sclerotia were noticed in the leaf sheaths of the plants from the infected plot. The presence of the sclerotia did not mean any disease, however, for none of the symptoms was seen at any time.

VI. ASSOCIATION OF *S. ORYZAE* WITH THE RICE CROP IN OTHER PARTS OF INDIA

The negative results of pot and field experiments led to the diseased plants being looked for in the fields at Pusa and elsewhere. The Economic Botanist in charge of rice investigations at Dacca (Bengal) wrote that the rice disease at that place had been definitely traced to borers which attacked and killed the plants. In the straw of such plants the sclerotia of the fungus invariably appeared. He was of the opinion that *S. oryzae* may not be the initial cause of the disease and that if it did play any role at all, it was of a secondary character.

The Government Mycologist at Coimbatore (Madras Presidency) stated that *S. oryzae* had been isolated on several occasions from rice straw but that it may be, if at all , a weak parasite and caused no serious damage to the crop in that presidency.

The Associate Professor of Botany, Lyallpur, wrote that in the Punjab the disease was doing a good deal of damage. The Director of Agriculture, Kashmir, reported in 1933 that the rice crop in the State showed unusual sterility and that he considered that it may be due to some fungous disease. The Rice Specialist,

Bihar and Orissa, who has extensively toured in the rice tracts of the Province and who has the crop under close observation at Sabour, stated that no sclerotial disease as described by Butler or Shaw was noticed by him anywhere in the Province, nor had he seen any stem-rot.

A watch for this disease has been kept in the rice fields at Pusa and the neighbourhood. The disease has not been observed during the last three seasons though the harvested rice straw has shown the presence of the fungus in some cases.

In 1932 Dr. M. Mitra visited the Rice Breeding Station at Karjat, near Bombay. The Crop Botanist who has this crop under close observation had not observed any stem-rot disease that could be traced to fungi nor had he noticed any unusual sterility or tillering in his plots. But rice stubble left in his plots showed infestation by the fungus as also the rice straw from the same fields. It was manifest that the fungus was there in the soil and may have even attacked living plants but it evidently did not produce any disease.

In 1933 the writer visited Anakapalle, Samalkota, Maruteru, Coimbatore and Pattambi, all agricultural experiment stations in the Madras Presidency. The Superintendents in charge of these stations stated that there was no sclerotial disease of rice at these places and that the crop every year was normal. At Anakapalle the straw and stubble from plots that had just been harvested showed the presence of the fungus. The panicles were heavy with grain but the leaf sheaths were full of sclerotia. Evidently the crop had not suffered from sterility even though the fungus was present.

At Maruteru the writer was shown several plants which showed excessive tillering. Seven such plants were uprooted and dried. Three plants showed infestation by the fungus when examined at Pusa and four did not. At Maruteru itself several such plants failed to show any infestation by sclerotial fungi.

Such plants were also a feature at Pattambi. Four plants were brought to Pusa and examined but there was no trace of any sclerotial fungi. It further appeared that this fungus was absent at this farm, for, several samples of straw from this station, that were brought over for examination, failed to show any sclerotial infection.

At the Coimbatore Rice Experiment Station, sclerotial infestation of standing plants as well as harvested plants was noticed. There was not any sterility or excessive and abnormal tillering. The investigators at this place had not observed the stem-rot symptoms either. The only disease that was causing concern was the foot-rot due to *Fusarium moniliforme* Sheld, and this had been controlled to a large extent by seed treatment.

On the Karnal Farm in the Punjab the writer examined several healthy plants with heavily filled panicles. Some of these showed sclerotia in the leaf sheaths but the plants could under no circumstances be considered as being diseased. No where were any plants showing the stem-rot symptoms as described by Reyes or Tullis and Cralley ever noted.

No opportunity to visit Western and Northern Punjab and Kashmir during the rice season presented itself but even though the specimens of rice plants sent by the Director of Agriculture, Kashmir, showed sterility, only two plants, out of seven plants received, showed infestation by the sclerotia. The others were free from it. The culture of *Sclerotium oryzae* received from the Punjab gave negative results in the experimental tests already cited in Table VII.

VII. Discussion of results

Data have been presented in the foregoing pages which seriously question the capacity of *S. oryzae* to produce disease in rice plants under conditions obtaining in some parts of India. The isolates used in the investigation were not only of Indian origin but several of them were received from pathologists abroad at whose hands they had proved to be pathogenic to rice plants, producing the well known stem-rot disease. On plants growing in water cultures in large size test tubes, all these cultures were equally parasitic and brought about a seedling disease.

In pot experiments extending over three years and comprising over 100 pots heavily infested with sclerotia of *S. oryzae*, this fungus has shown itself to be incapable of bringing about the disease at Pusa. The straw when harvested has shown the presence of the sclerotia in the tissues of the culms and the leaf sheaths, leaving no doubt regarding the presence of the infective material in the soil.

The field experiments conducted during the past three years have further confirmed the above observations. In two plots that were heavily infested with the fungus initially and again at intervals, no disease developed at any time. There was not any abnormal tillering or any marked sterility which was not a feature of the check plot plants as well. None of the plants showed any stem-rot and the few deaths that took place in the fungus-infested as well as check plots were definitely found to be due to termite attack.

The yield of the plots was not less than that of the check plot. In 1933 living plants at various stages of growth were occasionally examined to see if the fungus could be seen within the tissues. In late October, when the plants were about five months old it was noticed that in the tissues of leaf sheaths fungal hyphae which were sub-hyaline to brown in colour were present intercellularly and peculiar appressoria-like structures were also present. Just before harvest even sclerotia were noticed within the tissues. They appreciably increased when the plants were harvested and the straw was dried. There was however no disease and the plants bore well filled panicles.

It was apparent that the fungus was able to effect entry within the host plants but it evidently could not make enough progress so as to kill the plants or produce other disease symptoms as a result of this infection. It was noted that at the time the fungal hyphae were observed in the tissues, many of the older bottom leaves had dried up and possibly the fungus enters through such leaves. Such dead

leaves were a feature of the plants in the check plots as well but the tissues of leaf sheaths there did not show the presence of the fungus nor were there any sclerotia seen in the straw of this plot after harvest.

Field observations made at various places furnished further confirmatory evidence to show that while the fungus was able to infest the plants at a rather late stage of their growth, it was not able to bring about the disease. At many agricultural stations where the rice crop was under study the investigators were unable to detect abnormal tillering, sterility or stem-rot but in their plots left over stubble invariably showed the presence of the sclerotia which were also found in the harvested straw. These sclerotia were either of *S. oryzae* or *R. microsclerotia*.

Standing plants in the fields bore well filled panicles but at the base of the leaf sheaths sclerotia were invariably noticed. Sterile panicles also showed such infection. Presence of sclerotia was not invariably an indication of sterility. Rice straw from Pattambi was free from sclerotial infection and possibly this fungus does not occur there but, all the same, plants with abnormal tillers and with sterile panicles were present in the fields there.

Jones [1930] has shown that some types of rice plants show two to ten per cent sterility. This sterility increases when such types are hybridised, for in the hybrids between these the percentage of sterility in the F_2 has been as much as 48. Jones has ascribed this sterility to incompatibility in the chromosome mechanism. It is possible that to some extent the sterility seen in the rice plants may have been due to such a cause.

Taking all the above facts into consideration, it is permissible to ask whether *S. oryzae* is at all pathogenic to rice and if it is, how it can occur in association with its host without causing the disease which it is known to bring about elsewhere.

That it is a parasite of a rather virulent nature in Italy, Indo-China, the Philippine Islands and the U. S. A., is clear from the reports of pathologists in those countries. It is also manifest from the investigations of Park and Bertus [1932] that the fungus causes a seedling disease in the crop and possibly abnormal tillering in mature plants in Ceylon. But in India different conditions of environment prevail and even the nature of the soil is different. Though the fungus evidently attacks the plants because its presence in living plants in the basal tissues has been noted, it does not cause any appreciable effect on the growth of the host or injure it by bringing about stem-rot. That such a phenomenon is possible is apparent from several instances that can be cited. *Endothia parasitica* (Murr.) Anderson et Anderson is not a parasite of any consequence to chestnuts in Japan where the fungus is endemic. But this fungus has wiped out the chestnut trees on the Atlantic Seaboard of the United States of America. Similarly in the case of the *Fusarium* wilt disease of cotton, acclimatised American cottons are immune to wilt due to *Fusarium vasinfectum* Atkinson, in wilt sick soils in India. Indeed an investigation by the writer has shown that even American cotton varieties that

are highly susceptible to this disease under American conditions are immune under Indian conditions. The nature of the soil and the environment play therefore an important role in producing disease in plants by soil-inhabiting fungi.

Park and Bertus [1932] state that in Burma, *S. oryzae* is very common in paddy fields but does not cause any serious disease in the rice crop. Even with their successful pot experiments they state that under normal conditions of growth, parasitism of this fungus is not very marked, but the conditions favouring the disease do not seem to be very clear to them.

There is nothing to show, however, that when the host is introduced in a new country, the same parasite may not become a highly destructive factor against the successful cultivation of the crop. But in the country where the crop has grown for thousands of years, the parasite and host evidently lie side by side without the former causing disease in the latter.

Because of these considerations and the experimental evidence that has been adduced, the conclusion is irresistible that *Sclerotium oryzae*, while attacking living plants, does not produce any disease symptoms under normal conditions in the rice crop in India.

VIII. Summary

1. Important differences in the symptoms of rice diseases due to *Sclerotium oryzae* as described by different investigators are pointed out.

2. Cultures of spp. of *Sclerotium* assembled from various sources could be grouped into four categories, and this paper deals with the morphology and parasitism of two of them.

3. One *Sclerotium* possessed coloured hyphae, small smooth sclerotia and had the ability to change the colour of some substrates and was identified as *S. oryzae*. The cultures produced conidia of *Helminthosporium sigmoideum* but not the ascigerous stage. By analogy, however, it is considered that this fungus is *Leptosphaeria salvinii*.

4. The other (? *S. oryzae* non-chromogenic strain of Park and Bertus)* had hyaline mycelium, larger sclerotia with a rough surface, was unable to change the colour of substrates and was identified as *Rhizoctonia microsclerotia*.

5. *S. oryzae* failed to bring about any disease in pots or fields heavily infested with sclerotia. Sclerotia were however noted in the leaf sheaths and culms after the crop was harvested. In tests conducted in heavily infested soils negative results have been invariably obtained. In test-tube experiments, however, the fungus was able to produce the disease.

6. Pot tests with *R. microsclerotia* also gave negative results.

* See Appendix.

7. *S. oryzae* occurs in rice fields and the sclerotia can be seen infesting the sheaths and culms of healthy plants. Some plants with excessive tillers had sclerotial infestation while a large number did not have such infection.

8. Sterility could not be correlated with the presence of this fungus for both sterile and healthy plants showed infection and at the same time a large number of sterile plants did not show any infection.

9. The conclusion is therefore drawn that under normal Indian conditions these fungi are unable to produce disease in the rice crop.

IX. Appendix

Since the manuscript of this paper was finally prepared, Park and Bertus have published further results of their investigations on the sclerotial diseases of rice in Ceylon (*A. Roy. Bot. Gard. Peradineya*, 12 : 1-36, 1934). In part 3 of this communication they deal with a disease due to a strain of *Rhizoctonia solani* Kuhn which they designate ' A strain ', for it displayed certain constant differences from *R. solani*. This fungus has no bearing on any of the sclerotial fungi included in this paper. In part 4 they deal with *Sclerotium oryzae*, A strain and in part 5 with another *Rhizoctonia* which they call *R. solani*, B strain. A perusal of their paper and an examination of the plates revealed the fact that of the *Sclerotium* spp. sent by Park, the one considered in this paper as *S. oryzae*, A strain, non-chromogenic, was really their *R. solani*, B strain.

Their observations with regard to the growth in culture of this fungus and the sclerotial measurements are in accord, in major respects, with those obtained here. The only characteristic which this last named fungus has in common with *R. solani* is the mode of branching and the constriction at the junction of the branch with the parental hyphae. Typical *R. solani* discolours the medium when grown on potato-dextrose agar, giving the substrate a brownish tinge. Isolates No. 8, 9, 10 and 11 do not do so and the medium on the contrary retains its original tint. The sclerotia of *R. solani* are furthermore diffuse and crust like, while those of this fungus are compact, definite in form and oval to globose in shape. The cells of the medulla are also globose in the case of this rice *Rhizoctonia* and do not show the branched hyphal character of the medullary cells of *R. solani*. Park and Bertus do not seem disposed to consider this as a species different from *R. solani* as it differs only in a few respects and they prefer to call it B strain of it. But on the suggestion of Mr. Ashby this fungus has been identified here as *Rhizoctonia microsclerotia* Matz which identification has been confirmed by Matz himself. It is manifest therefore that *Rhizocotonia solani*, B strain of Park and Bertus is *R. microsclerotia* Matz.

As regards the pathogenicity of this fungus, Park and Bertus failed to induce it to parasitise rice seedlings growing in test tubes. In their pot experiments they state that only four out of a total of twenty-two plants died. It should be noted that the plants were not completely killed as new tillers were put forth by the plants, that only the central shoot had succumbed and that the tests were carried out in unsterilised soil, so that the parasitism of the fungus cannot be considered as settled.

They admit that the fungus is weakly parasitic and that it is not clear to them whether under field conditions it was responsible for the two outbreaks that had been reported, infection being absent in subsequent crops raised in the same fields.

The description and the sclerotial measurements of the fungus, which they have described as *S. oryzae*, A strain, tally with those of an isolate which was received by the writer from Nakata under the name *Sclerotia microsphaeroides* Nakata. Park and Bertus' *S. oryzae*, A strain does not have any of the characteristics common to all the different strains of *Sclerotium oryzae*. It has sclerotia which are not easily separable, with rather rough surface and with a much smaller size, and in these respects it agrees perfectly with Nakata's *Sclerotium*. Furthermore as *S. oryzae* has now been shown to be a stage of *H. sigmoideum* and *L. salvinii*, a sclerotium differing from this to the extent *S. oryzae*, A strain does, cannot be considered as a strain of that fungus. Perhaps the name given by Nakata, *Sclerotium microsphaeroides* would be more appropriate.

X. Acknowledgments

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SOME OBSERVATIONS ON THE ESSENTIAL OIL CONTENT OF *ROSHA* GRASS (*CYMBOPOGON MARTINI* VAR. *MOTIA*)

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(With three text-figures)

Rosha grass on steam distillation yields an essential oil known as Palmarosa oil or Rusa oil which is used as a scent for soaps and also in the manufacture of various perfumes. About 230 acres of land near Jaranwala (Punjab) are under this crop. The grass was planted about eight years ago ; and the crop at about six inches above ground level is cut annually for distillation in the winter (October to December). The part remaining above ground is burnt and a fresh crop sprouts in the month of March.

In past years it was observed that if the crop was not distilled before the onset of frost, the oil content was seriously affected and it seemed likely that cutting the grass and stacking it before the appearance of frost might arrest the decline in the oil content. The investigation here described was therefore started with the object of determining the amount of oil in different parts of the plant, leaves, flowers, and stalks ; the effect of frost on the seasonal variation of the oil content in different parts, and the effect on the oil content of harvesting and stacking the crop before frost.

The investigation lasted from August 1932 to March 1933.

Material and methods of analysis

I. MATERIAL AND SAMPLING

Half an acre of grass planted four years previously was reserved and a composite sample obtained from twenty to thirty different places—carefully packed in a bag to avoid damage to leaves and flower-heads and also to minimise the loss of moisture—was brought to the laboratory for analysis. About eighteen hours elapsed between the sampling at the farm and the time of analysis. Leaves, stalks and flower-heads were carefully separated, cut into small pieces, and kept separately after thorough mixing. Another portion consisting of the entire plant was similarly treated.

II. Methods of analysis

(*a*) *Dry weight*

Individual samples as obtained above were dried to constant weight at 100°C. for 24 hours.

(*b*) *Essential oil*

Samples of leaves, stalks, flowers and the entire plant from 100 to 800 grams in weight were distilled in steam for two hours, and the distillate received directly into a large separating funnel. The distillation was complete after about one hour and a half, when no more oil came over. The water which settled at, and was constantly removed from, the bottom of the separating funnel during the distillation wa turbid, and on shaking with ether gave only a negligible amount of oil, while the oily layer from the separating funnel was transferred to a small tared separating funnel. In order to remove traces of moisture a small test tube (made of thin tin foil) containing anhydrous sodium sulphate was kept suspended overnight in the funnel. This treatment dried the oil to a constant weight. The dried oil was then weighed. The percentage of oil given in the following tables has been calculated on the dry weight of the material analysed.

(*c*) *Chemical and physical examination of the oil*

The analysis consisted of the following determinations :—

1. Specific gravity.
2. Refractive index.
3. Optical rotation.
4. Solubility.
5. Acid value.
6. Ester value.
7. Ester value after acetylation.
8. Free alcohol (as geraniol)
9. Total alcohol (as geraniol)

All the determinations were carried out after the manner of Gildemeister and Hoffmann [1913], which may be described briefly as below :—

1. Specific gravity.—Specific gravity was determined at room temperature with a pyknometer and the figures obtained reduced to 15°C. by applying a correction coefficient of 0·00075 per each degree of difference added or subtracted as the temperature was above or below 15° C. [Villavecchia, 1918].

2. Refractive index was determined by Abbe's refractometer at room temperature and corrected to 15°C. by 0·00035 factor for each degree of temperature [Gildemeister and Hoffman, 1913].

3. Optical rotation was determined by polarimeter in a one decimeter tube at room temperature.

4. Solubility.—Five to ten c.c. of the oil were placed in 100-c.c. stoppered graduated cylinders and alcohol (70 per cent by volume) was added in small portions, with vigorous shaking, till all the oil was dissolved.

5. Acid value, 6. Ester value.—To about two grams of accurately weighed oil in a 500-c.c. flask, about twice its volume of neutral alcohol was added. The mixture was carefully titrated with semi-normal potassium hydroxide solution using phenolphthalein as indicator. After the neutralisation of the free acid, 10 c.c. of half-normal solution of alcoholic caustic potash were added and the mixture heated on a water bath under a reflux condenser for one hour, and on cooling the excess of alkali titrated with semi-normal sulphuric acid. The acid and ester values were calculated from the first and the last titrations respectively.

7. Ester value after acetylation.—Ten c.c. of oil mixed with an equal volume of acetic anhydride and about two grams of dry sodium acetate were boiled under a reflux condenser for one hour, cooled, a small quantity of water added to the flask, and the mixture again heated for 15 minutes on the water bath to decompose the excess of acetic anhydride. The oil was separated in a separating funnel, washed with water until neutral and dried with anhydrous sodium sulphate. About two grams of the acetylated oil were saponified as above and the free acid having been previously neutralised, the ester value of the acetylized oil was determined.

8. Free alcohol, 9. Total alcohol (geraniol).—These were calculated from the ester values before and after acetylation of the oil from tables given in "The Volatile Oils" by Gildemeister and Hoffmann. While calculating these values, the ester in the original oil has been included.

Experimental results

A. Changes in the dry weight of different parts of the plant during growth and maturity

The dry weights of the entire plant and its different parts are given in Table I and graphically shown in Fig. 1. It will be noticed that the dry weight in all cases continues to increase upto the end of September when it shows a decline lasting till about the end of October when, synchronising with the appearance of flowers it suddenly rises to be followed by a gradual fall till the beginning of December. When, however, a severe attack of frost is associated with a sudden rise in the dry weight, which is more pronounced in the flower-heads, this rise in all the cases continues till the end of the season. Attack by frost rendered the entire crop brown, with consequent loss of moisture resulting in a higher percentage of total solids. This effect was more pronounced in the case of the flower-heads, probably owing to their being exposed directly to frost.

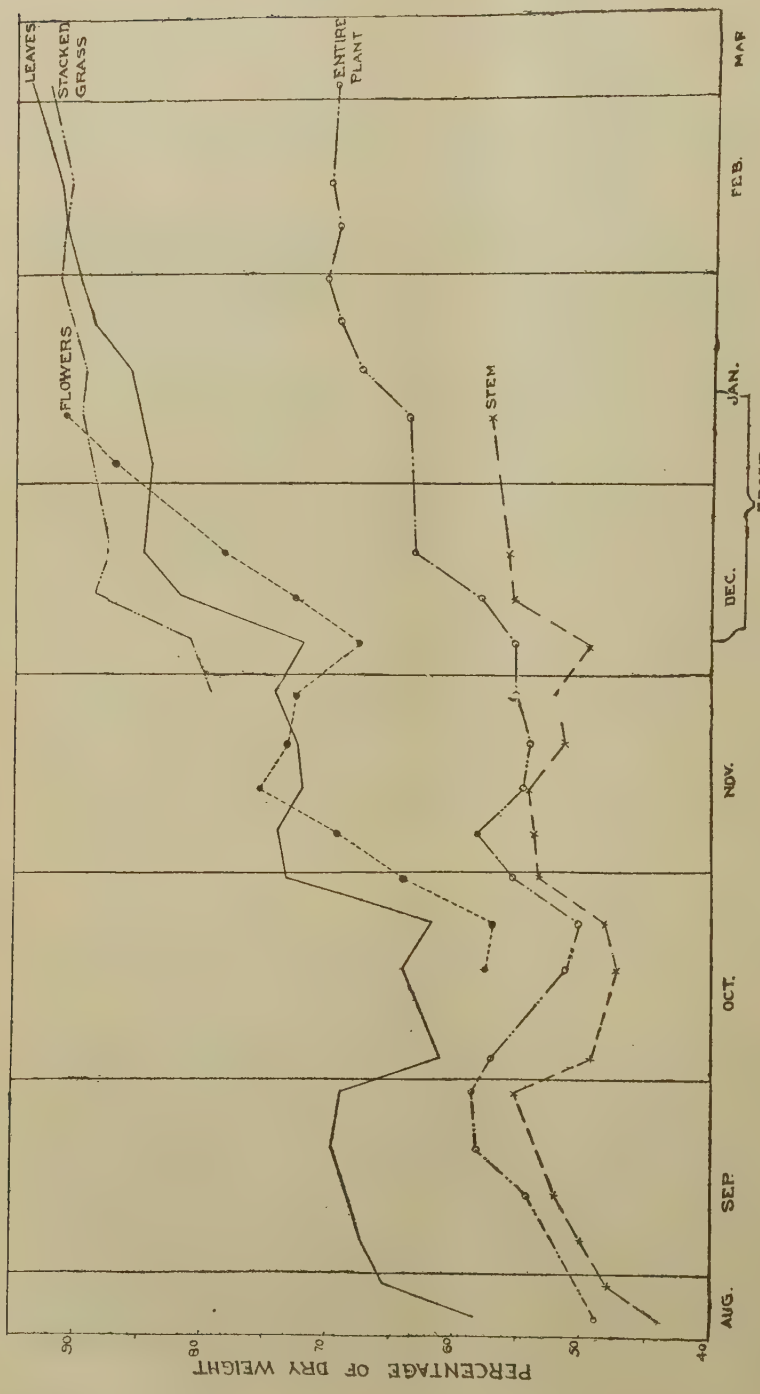

Fig. 1. Variations in dry weight of Rosha grass (*Cymbopogon Martini* var. *Motia*) throughout the season.

(B) Seasonal variations in the essential oil content of leaves, stalks, flower-heads and the entire plant

1. Essential oil in leaves

The essential oil data are given in Table I and graphically represented in Fig. 2. It is seen that the oil-content of leaves ranges between 1·22 and 1·30 per cent of the dry weight in the month of September, and gradually rises to a maximum of 1·39 per cent by the middle of October, when flowers appear, after which it shows a slow gradual decline till the end of November. After frost the oil content shows a further decrease, the net loss being 13·0 per cent of the maximum oil content. With a further advance in the season (by the beginning of March) the oil content gradually falls to a minimum of 0·77 per cent about the beginning of March, thus showing a decrease of about 45 per cent from the maximum.

2. Essential oil in flower-heads

Flowers appear in the second week of October and by the bginning of 3rd week 50 per cent of the plants were in flowers. The oil content of the flowers reaches a maximum of 1·37 per cent (on dry weight) by the end of October and then declines. The oil content shows a sudden decline after frost, a figure of 0·59 per cent by the second week of January when it falls still further, showing at this time a total decrease of 59 per cent from the maximum.

3. Oil-content in stalks

From Table I it is seen that the oil content in stalks is negligible, being only 0·03 per cent (on dry weight) during the month of September. Determinations of the oil content of stalks were discontinued from October onwards, as it was noticed that, with the advance of the season the stalks were thickening and becoming tough.

TABLE

Dry weight and essential oil contents of Rosha grass

Date	Dry weight per 100 grms. of fresh weight (Material dried at 100°C. for 24 hours)					Grms. of essential oil per 100 grms. fresh weight of the material analysed		
	Leaves	Stalks	Heads (Flower)	Entire plant	Stacked grass	Leaves	Stalks	Flower-heads
24th August 1932 .	58·4	44·0	...	48·8	...	0·722	0·011	...
29th August 1932 .	65·4	48·0	...	...	...	0·796	0·016	...
5th September 1932.	67·4	50·0	...	...	...	0·873	0·013	...
12th September 1932	63·6	52·0	...	54·2	...	0·821	0·018	...
19th September 1932	69·2	50·6	...	58·4	...	0·818	0·013	...
28th September 1932	69·0	55·2	...	58·2	...	0·829	0·014	...
3rd October 1932 .	61·2	49·2	...	57·4	...	0·807	0·014	...
17th October 1932 .	64·2	47·4	57·6	51·2	...	0·893	...	0·768
24th October 1932 .	61·8	48·2	57·0	50·2	...	0·853	...	0·780
31st October 1932 .	73·2	53·4	64·0	55·4	...	0·981	...	0·876
7th November 1932	74·0	53·6	69·2	58·4	...	0·915	...	0·864
14th November 1932	72·0	54·4	75·5	54·6	...	0·964	...	0·926
21st November 1932	72·4	51·2	73·0	54·2	...	0·948	...	0·874
28th November 1932	74·2	52·0	72·6	55·2	79·2	0·916	...	0·868
6th December 1932	69·8	55·4	67·4	55·2	80·6	0·864	...	0·788
13th December 1932	81·6	55·8	72·5	58·0	88·6	0·985	...	0·773
20th December 1932	84·6	51·2	78·2	63·3	87·4	1·037	...	0·879
3rd January 1933 .	84·0	57·4	86·8	59·2	84·6	1·087	...	0·956
10th January 1933 .	89·2	56·0	90·8	63·6	89·6	0·917	...	0·528
17th January 1933 .	85·6	...	89·2	67·4	89·2	0·899	...	0·510
25th January 1933 .	88·7	...	...	69·3	90·5	0·874	...	...
31st January 1933 .	89·8	...	...	70·2	91·4	0·815	...	...
8th February 1933	90·8	...	...	69·2	90·6	0·762	...	...
15th February 1933	91·2	...	...	69·8	90·2	0·715	...	...
2nd March 1933 .	93·6	...	...	69·4	92·2	0·718	...	...

I.

(Cymbopogon Martini *var.* Motia) *Season 1932-33*

Essential oil content calculated to 100 grms. dry weight of the material analysed							Remarks
Entire plant	Stacked grass	Leaves	Stalks	Flower-heads	Entire plant	Stacked grass	
0·155	...	1·236	0·025	...	...	...	
...	...	1·218	0·033	...	...	...	With the advance of season the leaves above the root were drying and becoming brown.
...	...	1·295	0·026	...	...	...	
0·148	...	1·291	0·035	...	0·273	...	
0·161	...	1·183	0·026	...	0·276	...	
0·164	...	1·302	0·025	...	0·282	...	
0·164	...	1·319	0·028	...	0·286	...	
0·198	...	1·391	...	1·334	0·386	...	About 50 per cent of the plants in the plot were flowering.
0·199	...	1·380	...	1·368	0·395	...	
0·222	...	1·340	...	1·369	0·400	...	Maturity period.
0·210	...	1·332	...	1·249	0·307	...	
0·202	...	1·338	...	1·228	0·370	...	
0·191	...	1·311	...	1·200	0·365	...	The crop was cut on 21st November and dried in field and stacked on 26th Nov. 1932.
0·192	0·237	1·289	...	1·246	0·348	0·300	
0·187	0·248	1·238	...	1·171	0·339	0·308	Severe attack of frost.
0·162	0·223	1·207	...	1·067	0·279	0·252	
0·189	0·220	1·226	...	1·125	0·299	0·252	Intermittent attacks of mild and severe frost.
0·179	0·215	1·294	...	1·101	0·302	0·254	
0·186	0·225	1·028	...	0·592	0·292	0·251	Intermittent attacks of frost.
0·183	0·219	1·050	...	0·562	0·272	0·246	
0·180	0·230	0·985	...	...	0·260	0·254	
0·171	0·242	0·907	...	...	0·244	0·265	
0·165	0·230	0·839	...	...	0·238	0·254	
0·157	0·248	0·785	...	...	0·225	0·275	
0·127	0·224	0·767	...	...	0·183	0·243	

4. Oil-content of the entire plant

Seasonal changes in the oil content of the entire plant are similar to those of the leaves and flowers. During the time preceding the maturity period (third week of October to end of November) the oil content in the plant ranges from 0·15 to 0·22 per cent on the fresh weight and 0·27 to 0·40 on the dry matter. The period of maximum oil content in the entire plant synchronises with that in the leaves and flowers. With frost during the first week of December a sudden fall — though not as great as in the leaves—in the oil content is observed. Later on with intermittent attacks of frost and advance of season the oil content shows a gradual decline. By the end of the second week of January, when frost ceased, a decrease of 32 per cent from the maximum was observed, whereas by the beginning of March the decrease was about 54 per cent from the maximum.

C. The effect on the oil-content of stacking the grass before frost

The fully matured crop from half the plot reserved for the investigation was harvested before the onset of frost on the 21st November, and in order to avoid fermentation during stacking, it was previously dried in the sun for four days. For the estimation of oil about thirty representative samples from the field were collected, carefully packed in muslin bags to avoid injury to the flower-heads and leaves, and placed in the above-mentioned stack of grass on the 26th of November. The oil content of these samples and of the corresponding standing crop in the field was periodically examined. The data of dry weight and essential oil content of the stacked grass are given in Table I and graphically shown in Figs. 1 and 2. As seen from Fig. 1 the dry weight shows a rapid increase till the middle of December, after which it decreases till the maximum figure is obtained by the beginning of March.

It will be seen from Fig. 2 that the loss in oil content in the stacked grass takes place only in the first two weeks of stacking after which the oil content remains practically constant over a period of three months when the analyses were discontinued.

Table II

Showing the comparison of the essential oil content of the standing Rosha grass in the field and the corresponding stacked grass

Date	Standing crop in the field				Stacked grass		
	Dry weight per cent I	Per cent essential oil on fresh weight II	Per cent of essential oil calculated on initial fresh weight III	Dry weight per cent IV	Per cent essential oil on fresh weight V	Per cent essential oil calculated on initial fresh weight VI	Loss of oil in the stacked grass (0·191—figures of the previous column)
21st November 1932	54·2	0·191	...	...	...	...	...
28th November 1932	55·2	0·192	0·188	79·2	0·237	0·162	·029
6th December 1932 .	55·2	0·187	0·184	80·6	0·248	0 167	·024
13th December 1932 .	58·0	0·162	0·151	88·6	0·223	0·136	·055
20th December 1932.	63·3	0·189	0·162	87·4	0·220	0·136	·055
3rd January 1933 .	59·2	0·179	0·164	84·6	0·215	0·138	·053
10th January 1933 .	63·6	0·186	0·158	89·6	0 225	0·136	·055
17th January 1933 .	67·4	0·183	0·147	89 2	0·219	0·133	·058
25th January 1933 .	69·3	0·180	0·141	90·5	0·230	0·138	·053
31st January 1933 .	70·2	0·171	0·132	91·4	0·242	0·143	·048
8th February 1933 .	69·2	0·165	0·129	90·6	0·230	0·138	·053
15th February 1933 .	69·8	0·157	0·122	90·2	0·248	0·149	·042
2nd March 1933 .	69·4	0·127	0·099	92·2	0·224	0·132	·059

Table II gives the oil content of the standing crop in the field, together with the corresponding figures for the stacked grass. Periodic determinations of the oil content of fresh and the stacked grass have been calculated on the intial fresh weight of the green crop as it was noted on 21st October, the date of cutting the crop for stacking. Thus figures of columns III and VI are the actual amounts of oil per 100 grams of intial fresh weight of the green and stacked grass respectively. It is clear from the figures of these two columns that the standing crop in the field, though subjected to intermittent attacks of frost from the second week of December to the middle of January, contains essential oil in excess of that in the stacked grass uptil the 25th of January 1933 and thereafter the stacked grass maintains a higher oil content than the crop standing in the field. In order to see whether the differences in the essential oil content of the standing and the stacked grass are significant, Fisher's [1930] 't'-test of significance has been applied to the figures of columns III and VI (upto the 25th of January 1933). The observed value of 't' is 6·65 (while the one per cent value of 't' for $n = 7$ is only 3·499) showing that the differences in the oil content of the standing green grass and the corresponding stacked grass are clearly significant. It is evident, therefore, that

the standing crop, though affected by frost yielded more oil than the stacked grass till the end of January, whereas due to the advance of the season and adverse climatic conditions, the standing crop dried up with a considerable decrease in the oil content, which in the month of February was only 84 per cent of the oil present in the stacked grass at this time.

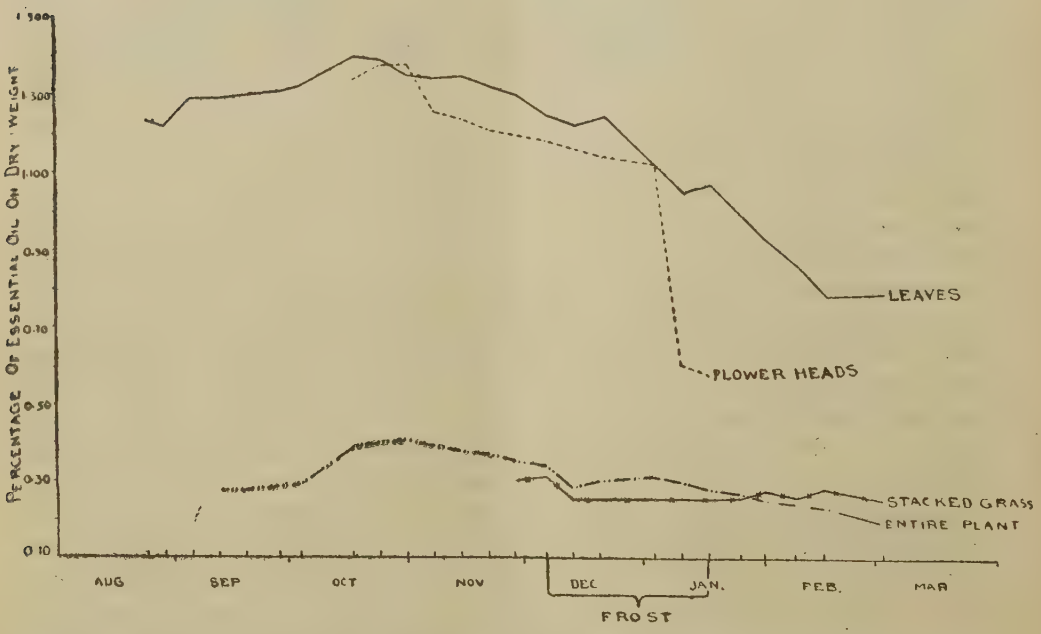

Fig. 2. Seasonal variation in the essential oil content of Rosha grass (*Cymbopogon Martini* var. *Motia*) dry weight basis

The loss in oil-content of the grass during the period of stacking is shown in Fig. 3 and Table II. The values in column VII have been plotted against time. From the table it is seen that the stacked grass shows a decrease in the oil content from 0·191 (oil present in the grass on the day on which it was harvested for stacking) to an average figure of 0 164 uptil the 28th of November, showing a decrease of 14 per cent and a further decrease of about 17 per cent in the oil content till the 6th of December after which it remained almost constant till the beginning of March. These points are further illustrated by a study of Fig. 3 from which it is seen that a rapid loss in the oil content of the stacked grass occurred till the end of the first week of December and thereafter it maintained almost a constant level with only slight fluctuations (due probably to experimental error) till the end of the period of stacking.

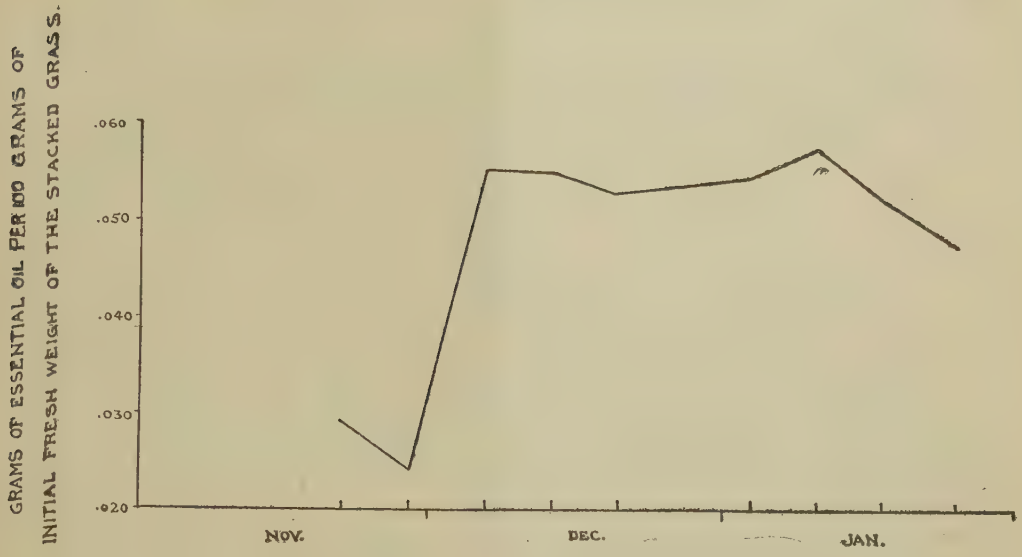

Fig. 3. Loss of essential oil in the stacked grass

D. Chemical composition of palmarosa oil

Table III gives analyses of oil obtained from different sources. No appreciable variation in the constants of the above samples is observed except in the acid value, the ester value and the geraniol content. It will be seen that with the exception of sample 2 all the remaining samples gave a high acid value; sample 3 giving the highest figure. The high acid and ester values in sample 3 indicate a greater stability of esters and free fatty acids during the process of steam distillation as carried out in the laboratory, thus resulting in a slightly lower content of free geraniol and a comparatively high content of total geraniol. It is clear from the free and total geraniol figures (93·5 to 96·06 per cent respectively) that the leaves yielded an oil of the best quality, while the stacked grass gave the poorest quality oil. The oil obtained both from stalks and flower-heads (which contains mostly the woody stalk) is almost similar in quality.

TABLE III

Physical and chemical constants of palmarosa oil obtained from different sources

Sample	Specific gravity at 15°C.	Refractive index at 15°C.	Optical rotation	Solubility Vol. of alcohol (70 per cent. vol.) dissolving one volume of oil	Acid value	Ester value	Ester value after acetylation	Free alcohol (geraniol) per cent	Total alcohol (geraniol) in the original oil per cent
1. 1931. Farm distilled	0·8884	1·4789	+0·025	1·45	1·13	13·43	272·67	88·50	92·19
2. 1932. Farm distilled .	0·8887	1·4789	+0·030	1·52	0·65	12·39	274·85	89·86	93·22
3. Laboratory distilled (1932).	0·8931	1·4790	...	1·60	1·57	27·93	283·83	87·09	94·78
4. From leaves Farm distilled, 1932.	0·8891	1·4781	+0·005	1·41	1·11	9·33	280·28	93·52	96·06
5. Oil from flower-heads (1932) Farm distilled.	0·9001	1·4780	+0·030	1·36	1·24	10·45	276·86	91·56	94·43
6. Oil from stalks (1932) Farm distilled.	0·8895	1·4781	+0·005	1·39	1·04	14·28	279·62	91·10	95·03
7. Oil from stacked grass (1932) Farm distilled.	0·8877	1·4791	+0·010	1·50	1·07	15·09	272·73	87·82	91·98

N.B.—In calculating the amount of total and free geraniol the esters in the original oil have been taken into consideration.

Discussion

It is evident from the data presented in the preceding pages that the leaves yield the highest and the largest amount of oil throughout the season.

The flowers (appearing in the third week of October) show a maximum oil content during the fourth week of October, a period which is associated with the maximum of oil content both in the leaves and in the entire plant. Thus the grass is fully mature during the fourth week of October, whereas the oil content in all cases (leaves, flower-heads and the plants) showed a slow gradual decrease during the month of November. The onset of frost, which followed, affected the oil content to a marked degree. These observations regarding the essential oil content of the plant during growth and maturity (before the attack of frost) are in accordance with the work of Reilly and Taylor [1927] and Hogstad [1926] who worked on the "Oil of Peppermint"; and "The Origin, Nature, and Physiological Roll of the Essential Oil of American Wormwood," respectively. These workers have also found that the highest yields of oil are obtained during the period of flowering, and also that the largest amount of oil is obtained from the leaves and flowering tops which show a gradual decrease in the oil content as the plant reaches maturity.

The most suitable time for distilling the oil seems to extend from the fourth week of October to the third week of November, a period which corresponds with the maximum oil content of the plant.

In regard to the effect of stacking on the oil content, as has been already shown, the percentage decrease up to the first week of January is less in the case of the standing crop, and removal and stacking of the crop before frost does not arrest the decline in the oil content. It is, therefore, suggested that to obtain a maximum yield of oil the crop should be harvested and the oil distilled before the frost.

V. Summary

Seasonal variations in the oil content of *Rosha* grass (*Cymbopogon Martini* var. *motia*) and its different parts, *viz.*, leaves, flowers and stalks have been studied from August 1932 to March 1933.

The leaves yielded the largest amount of oil throughout the season. Flowers are short-lived and gave the maximum amount of oil (1·37 per cent on dry weight) during the fourth week of November (a week after their appearance), the stalks contain only a negligible amount of oil (0·03 per cent on dry weight).

The whole plant yielded the maximum amount of oil about a week after flowering. The time extending from the fourth week of October and the third week of November has been found to be the ' maturity ' period and best suited for harvesting the crop and distillation of the oil.

Intermittent attacks of frost render the plant brown and decrease the oil content of the leaves, flowers and the entire plant to the extent of 13 per cent, 59 per cent and 32 per cent, respectively. The flower-heads are more susceptible to frost.

Stacking the grass before frost does not arrest the decline in the oil content. The crop, for distillation purposes, should therefore be harvested before the onset of frost.

Chemical examination of the oil indicates that the leaves yield the best quality oil. The flower-heads (containing the woody stalk), and the stalks give an oil of a slightly inferior quality, whereas the stacked grass yields the poorest quality oil.

The writer wishes to thank Dr. P. E. Lander and Dr. Ramji Narain for their valuable criticism and advice, during the course of the investigation.

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STUDIES ON *RHIZOBIUM LEGUMINOSARUM* OF BERSEEM (*TRIFOLIUM ALEXANDRINUM*)

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Introductory

Because of its high yields and great nutritive value berseem or Egyptian clover is becoming increasingly popular in the Punjab as a valuable fodder.

It was found, however, that it will not do well at all places and investigations showed that this was due to the lack of nodule formation on the roots. The defect was remedied by artificial inoculation of such soils with the requisite bacteria [Sarkaria, 1933] and the treatment was instrumental in increasing the yields about 86 per cent on the average*.

In view of the importance which this fodder crop has assumed in the Punjab, a study of its root-nodule bacteria and an investigation into their cross inoculation relationship with the nodule bacteria already present in our soils is of deserving importance.

Nobbé and his co-workers [1895] came to the conclusion that the bacteria in the nodules of the various legumes were all strains of the same organism. It was soon recognised however that different leguminous plants require specific organisms for inoculation.

Burrill and Hansen [1917] by a series of cross inoculations demonstrated the existence of eleven kinds of nodule bacteria and divided these into three distinct groups.

Later, Löhnis and Hansen [1921] studied the nodule bacteria from fourteen different legumes and divided their fifty-seven cultures into two groups which differed morphologically as well as physiologically.

Fred and Davenport [1918] established differences in the critical pH for the growth of the nodule bacteria of the common legumes and arranged them into five groups.

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* The average has been taken from the results of the inoculation experiments conducted at ten different places in the Punjab.

As a result of cross-inoculation studies Hansen [1921] divided the nodule bacteria of the various legumes into eleven distinct groups. The different members in any one group are those which can be inoculated by the strain of the nodule organism specific for that group. According to him all the *Trifolium* species are included in one group. The organism infecting the members of this group has been described as peritrichic.

On the basis of serological investigation, Klimmer and Kruger [1914] formed nine groups of legume bacteria. Later Bialosuknia and Klott [1923] noted that the same kind of plant from different sources may have nodule bacteria with different agglutinating properties. They also report that different nodules on the same plant may contain serologically different bacteria. This observation was later confirmed by Wright [1925] in the case of the soybean organism.

As will be clear from the above we have not yet been able to establish any morphological differences amongst the various groups of the nodule bacteria, except the division into two groups suggested by Löhnis and Hansen [1921]. We do not know whether we are dealing here with different species or mere biological races [Waksman, 1926].

For specific separation one has to depend on cross-inoculation and serological tests.

The agglutination test, which has been widely used in other fields of bacteriology for diagnostic purposes, has clearly shown that the nodule bacteria from even one legume could be separated into several groups. Hence in the present investigation only cross-inoculation and cultural methods have been used.

In addition to the cultural study of the four strains of the organism on some of the more important media, the effect of certain physical factors has also been investigated.

Experimental

Description of the organism

The organism is a gram-negative coccus or a rod, according to the stage in the cycle of its growth, varying from 1 to 3 μ in length and being about .1 μ in breadth. The organism when motile has one polar flagellum.

The organism studied was obtained from known cultures of bacteria isolated from the root nodules of berseem. These cultures had been tested for purity and ability to form nodules. All the cultures were further tested for the absence of *B. Radiobacter* on milk and potatoes according to the method of Löhnis and Hansen [1921].

The strains studied were freshly isolated from plants grown in different fields. For the cultural study four strains were used, while the other part of the investigation was mainly carried on with strain I.

Cultural characteristics

The cultural characteristics of the four strains were studied on a variety of different media. The rate and amount of growth were recorded from time to time. Besides nitrogen-free media a number of media containing nitrogen in addition to the various carbohydrates were also used since, contrary to the views previously held, it has now been shown by certain workers [Albrecht, 1920] that nodules are produced, and a satisfactory growth of the legumes does result, even when large amounts of organic nitrogen or nitrates are present in the soil.

The carbohydrate media used were prepared on three bases. Some were prepared on Ashby's solution as a base, others on soil extract and still others with beef extract broth. This was done with the object of providing no nitrogen (as in Ashby's) providing slight amounts of nitrogen (as in soil extract) and providing a good amount of nitrogen (as in beef extract).

The only differences noticed were in the rate and amount of growth as shown in Table I.

TABLE I

Showing cultural characteristics of the four strains. Media inoculated on 13th December 1931 and examined on 23rd December 1931.

Medium	Strain I			Strain II		
	Beef extract	Soil extract	Ashby's *	Beef extract	Soil extract	Ashby's
Glucose agar	+ + +	+ + +	+ +	+ +	+ +	+ +
Glucose broth	Slight haziness	Hazy growth	Slight haziness	Slight haziness	Hazy growth	Slight haziness
Lactose agar	+ + +	+ +	+	+ + +	+	+
Lactose broth	Slight haziness	Hazy growth	Slight haziness	Slight haziness	Hazy growth	Slight haziness
Saccharose agar	+ + + +	+	+ +	+ + + +	+	+ +
Saccharose broth	Very slight reddening	Haziness, colour lightened	Slight haziness	Indicator colour lightened	Haziness, colour lightened	Slight haziness
Dextrin agar	+ + +	+	+	+ + +	+	+
Dextrin broth	Slight haziness	Colour deepened pink	Hazy growth	Slight deepening of the colour	Colour lightened	Hazy growth
Litmus milk	Serum zone on the top			Serum zone on the top. litmus deepened blue		Colour of

* Calcium carbonate had been added to the Bouillon cultures and so the change in indicator could not be studied.

\+ Slight growth
\+ + Good growth
\+ + + Heavy growth
\+ + + + Extra heavy growth
— No growth

TABLE I—(*contd.*)

Medium	Strain III			Strain IV		
	Beef extract	Soil extract	Ashby's *	Beef extract	Soil extract	Ashby's
Glucose agar	+ + +	+ +	+	+ + +	+ +	+ +
Glucose broth	—	Haziness, good growth	Slight haziness	Slight haziness	Good growth	Haziness
Lactose agar	+ + +	+	+	+ + + +	+	+
Lactose broth	Slight haziness	Hazy growth	Slight haziness	Slight haziness	Good growth	Very slight haziness
Saccharose agar	+ + + +	+	+ +	+ + + +	+	+ +
Saccharose broth	Slight haziness	Hazy, colour lightened	Slight haziness	Slight haziness	Growth, colour lightened	Slight haziness
Dextrin agar	+ + +	+	+	+ +	+	+ +
Dextrin broth	Slight haziness	Colour lightened	Haziness	Slight deepening of the colour	Growth	Slight growth
Litmus milk	Serum zone on the top. Colour of litmus deepened blue			Serum zone on the top. Colour of litmus deepened blue		

* Calcium carbonate had been added to the Bouillon cultures and so the change in indicator could not be studied.

Since the growth in broth cultures was not definite in all cases, transfers from these cultures on to soil-extract-mannite-agar slants were made with the following results :

Inoculated from		Growth			
		Strain I	II	III	IV
Glucose broth	Beef extract	+ve	—ve	Scanty	Scanty
	Soil extract	+ve	+ve	+ve	+ve
	Ashby's	+ve	+ve	+ve	+ve
Lactose broth	Beef extract	+ve	Scanty	Scanty	Scanty
	Soil extract	+ve	+ve	+ve	+ve
	Ashby's	+ve	+ve	+ve	+ve
Saccharose broth	Beef extract	+ve	+ve	Scanty	Scanty
	Soil extract	+ve	+ve	+ve	+ve
	Ashby's	+ve	+ve	+ve	+ve
Dextrin broth	Beef extract	+ve	+ve	Scanty	Scanty
	Soil extract	+ve	+ve	+ve	+ve
	Ashby's	+ve	+ve	+ve	+ve

+ve signifies ' growth '.
—ve signifies ' no growth '.

Scanty growth on the slants indicates the unsuitable nature of the broth medium in which the organism had existed under unfavourable conditions.

From the above the following conclusions can be drawn :

1. That for all the four strains tested the growth in agar cultures was the best on carbohydrate media with beef extract base.
2. That the growth on saccharose beef extract agar medium has been the best for all the four strains ; being followed in order, by lactose, glucose and dextrin, the last giving the poorest results.
3. Only a slight growth was noticed in all the broth cultures and of these the cultures on soil extract base proved to be the best.
4. A serum zone was visible in litmus milk for all the four strains, an increase in the blue colour of the litmus indicator showing the alkaline nature of the by-products formed.
5. There was no growth on potato for any of the strains.

Reaction tolerance of the organism

In order to test the reaction sensitiveness of the organism, soil extract mannite agar [Joshi, 1920] with pH varying from 3 to 10 was prepared, slanted and inoculated in duplicate from a week old culture.

On examination after a week's incubation, good growth of the organism was observed in media slants with pH 5·0, 5·4, 5·8, 6·0, 7·2, 8·0, 9·0 and 9·8. Growth, however, was absent in tubes with pH 3·0 and 4·0. This showed that the organism could tolerate an acidity corresponding to pH 5·0 but not further.

Thermal death point

(*i*) The thermal death point of the organism was determined by inoculating mannite solution tubes of an approximately equal bore. Each of the tubes contained 10 c.c. of the solution. The inoculated tubes were exposed in duplicate to different temperatures for ten minutes. The temperature range studied was from 46°C. to 70°C., with a two-degree step. The results are given below :—

Strain No.	Lowest temperature at which no growth occurred
I	54°C.
II	52°C.
III	52°C.
IV	52°C.

(*ii*) Since the organism has its natural habitat in the soil it was considered desirable to determine the temperature at which it would cease growing in soil. Some sterile soil was mixed with an emulsion of the organism in a sterilized dish, with sterilized spatulas and in an inoculation cabinet. The soil so prepared was transferred in five-gram portions to each of the sterile culture tubes of equal bore through a sterile funnel.

The thermal death point in soil was determined by exposing these soil tubes (in duplicate) to various temperatures. The exposure was for 10 minutes at each temperature and the temperature ranged between 46°C. to 70°C. with an interval of two degrees. The tubes immediately after exposure were immersed in a bath of cold water and transfers made to soil extract mannite agar slants from each.

After making the transfers the exposed tubes were further incubated to see if any chance organism might have been left undetected. The results obtained were as follows :

Strain No.	Lowest temperature (°C.) at which growth was absent
I	54°
II	54°
III	54°
IV	54°

Transfers made after a fortnight's incubation from the soil tubes exposed to temperatures beyond 52°C. did not show any sign of growth on a selective medium.

(*iii*) The author has noticed elsewhere [Madhok, 1934] that the berseem organism from fully grown soil cultures, could survive an exposure for eight hours to the sun-light at a temperature up to 63°C.

With this idea in view, some sterile soil flasks were inoculated with an emulsion of the organism, stirred thoroughly with sterile spatulas and then incubated to get the maximum growth possible. At the end of a week these soil cultures were re-stirred in order to break up any lumps, and five-gram portions transferred to sterile plugged tubes of equal bore.

These tubes were exposed to different temperatures (10 minutes' exposure at each temperature) in a water bath and transfers made to soil extract mannite agar slants after each exposure. The examination of the transfers after four days showed that the organism in these cultures had survived at a temperature as high as 75°C.

Radial movement in soil

Since the nodule organism invades the roots of the host plant only in the " swarmer " or the motile stage, it was considered of interest to find the rate of movement of the berseem organism in soil and thus to get an idea of the spread of the organism from the point of inoculation.

For this purpose a technique similar to that used by Thornton and Gangulee [1926] for the lucerne organism was adopted, but, contrary to their observations, it was found that the berseem organism travelled very slowly in soil.

Observations were made on the soil contained in Petri dishes.

Experimental

(*i*) Ordinary loam soil with optimum moisture content was packed with hand in Petri dishes of about 6-in. diameter. The Petri dishes so packed were sterilized for two and a half hours at a pressure of 20 lbs. On cooling a little hole was made in the centre of the Petri dish by removing the soil with a sterilized cork borer. The hole so made was filled with sterile sand.

The inoculum was given by adding a couple of drops of the suspension of a week old culture on to the sterile sand in the centre, care being taken to add culture suspension just sufficient to moisten the sand up to the periphery.

After the lapse of one, two or four days a boring was taken (in triplicate) at different distances from the centre by means of a sterile cork borer and plated on soil extract mannite agar, with the results given below :

Incubation period (Days)	Distance from the centre		
	0 in.	½ in.	1 in.
1 . . .	+	— — —	— — —
2 . . .	+	— — —	— — —
4 . . .	+	— — —	— — —

+ means growth present.

— means no growth.

This operation was repeated with the same results showing that in a loosely packed soil (unpressed) the organism does not move even half an inch in four days

(*ii*) The above experiment was repeated with the addition of one per cent sugar and the following results were obtained :—

Incubation period (Days)	Distance from the centre		
	0 in.	½ in.	1 in.
1	+	— — —	— — —
9	+	— — —	— — —
14	+	— — —	— — —

Note.—After the expiry of 9 days a shining growth was noticed around the centre about ½ in. in diameter. However, on longer keeping it did not spread any further. It was probably the slime of the organism.

(*iii*) The addition of certain other carbohydrates to soil was also tried and the following results recorded :—

Incubation period	Soil treated with one per cent	Distance from the centre	
		½ in.	1 in.
9 days	Mannite	— — —	..
9 days	Sucrose	— — —	..
20 days	Mannite	+ + +	— — —
20 days	Sucrose	— — —	— — —

(*iv*) To see if the air spaces between the particles of the soil hindered the movement of the organism in soil, hard packing of the soil was tried against loose packing. It gave the following results :

Incubation period	Loose packed		Hard packed	
	½ in.	1 in.	½ in.	1 in.
15 days	— — —	..	— — —	..
20 days	..	— — —	+ + +	+ + +

(*v*) Another variation was made by the addition of 0·1 per cent dipotassium phosphate to the soil, since the compound has been known to increase the motility of the organism. The results were as follows :—

Incubation period	Soil moistened with 0·1 per cent K_2HPO_4		Soil without K_2HPO_4	
	½ in.	1 in.	½ in.	1 in.
10 days	— — —	— — —	— — —	— — —
14 days	+ + +	— — —	— — —	— — —

From the foregoing experiments it is clear that the organism is capable of moving in the soil only at a very slow rate. It spreads in the soil more through the agency of wind and water than by the help of its flagellæ.

Cross inoculations

In order to study the cross inoculation relationship of the organism, twenty-four glass tubes of about 8 in. length and 1½ in. bore were filled about three quarters each with a light loam soil and sterilized for 2½ hours at 20 lbs. pressure. These

were sown with berseem seed sterilized for five minutes in 1/500 mercuric chloride solution, and inoculated in triplicate with an emulsion of the nodule organism cultures isolated from gram (*Cicer arietinum*), *maina* (*Medicago denticulata*) *senji* (*Melilotus parviflora*), *methe* (*Trigonella foenum graecum*), lucerne (*Medicago sativa*), *shaftal* (*Trifolium resupinatum*) or berseem (*Trifolium alexandrinum*). Three tubes were left to serve as controls.

The cultures used in the experiment had been grown on soil extract mannite agar and were incubated at 30°C. for seven days before use. They had been tested for purity and their ability to form nodules on the host plants.

The tubes were kept on a laboratory table in diffused light* and the cotton plugs were removed only when the plants had developed sufficiently high. The roots were examined after about two months and it was found that only the organism of the *shaftal* nodules could inoculate berseem seed.

Test seed	Strain of the organism inoculated with	No. of nodules per plant	Remarks
Berseem	Gram organism	No nodules	
,,	,, ,,	,, ,,	
,,	,, ,,	,, ,,	
Berseem	Maina organism	No nodules	
,,	,, ,,	,, ,,	
,,	,, ,,	,, ,,	
Berseem	Senji organism	No nodules	
,,	,, ,,	,, ,,	
,,	,, ,,	,, ,,	
Berseem	Methe organism	No nodules	Root system very extensive
,,	,, ,,	,, ,,	
,,	,, ,,	,, ,,	
Berseem	Lucerne organism	No nodules	
,,	,, ,,	,, ,,	
,,	,, ,,	,, ,,	
Berseem	Shaftal organism	4, 3, 3, 6 : 4	
,,	,, ,,	7, 2, 2, 1 : 3	
,,	,, ,,	6, 4, 3, 3 : 4	
Berseem	Berseem organism	4, 2, 3, 6 : 4	
,,	,, ,,	3, 6, 8, 10 : 7	
,,	,, ,,	3, 2, 5, 6 : 4	
Berseem	Control	*nil*	Roots very extensive
,,	,,	,,	
,,	,,	,,	

* The column of soil in each tube was kept wrapped up in black paper during the course of the experiment.

In order to ascertain whether the berseem organism could reciprocally inoculate *shaftal*, the experiment was repeated in pots.

Eighteen pots of 4 in. diameter and about 4 in. height were each three parts filled with a light loam soil and sterilized for two hours at 20 lbs. pressure, on each of two consecutive days. Nine of these pots were sown with sterilized berseem seed and the other nine with sterilized *shaftal* seed. Of the nine berseem pots three were inoculated with an emulsion of the berseem organism and three with an emulsion of the *shaftal* organism. Similarly three pots of *shaftal* were inoculated with the *shaftal* organism and three with the berseem organism.

Three pots for each variety of seed tested were left to serve as controls, and were kept on the table in a green-house unexposed to wind or rain. Their roots were examined after two months when the following observations were made :—

Pot No.	Test seed	Inoculated with	Nodulation
1	Berseem	Berseem organism	Plenty
2	,,	,, ,,	,,
3	,,	,, ,,	,,
4	Shaftal	Berseem organism	Plenty
5	,,	,, ,,	,,
6	,,	,, ,,	,,
7	Berseem	Shaftal organism	Plenty
8	,,	,, ,,	,,
9	,,	,, ,,	,,
10	Shaftal	Shaftal organism	Plenty
11	,,	,, ,,	,,
12	,,	,, ,,	,,
13	Berseem	Uninoculated	*nil*
14	,,	,,	,,
15	,,	,,	,,
16	Shaftal	Uninoculated	*nil*
17	,,	,,	,,
18	,,	,,	,,

From the results obtained from these two sets of experiments it is clear that cross inoculations of berseem and *shaftal* are reciprocal.

Effect of ultra violet rays on berseem organism

Ultra violet rays are known to be injurious to bacteria but in an experiment conducted by us in this Laboratory the results [Madhok, 1934] showed that the injurious effect of the sun's rays on cultures of this organism on agar or in soil is

due to the rise in temperature of the medium, rather than to the intensity of the sun's rays in ultra violet radiations.

It was therefore considered necessary to test the effect of pure ultra violet light on agar and soil cultures of this organism. For this purpose a good growth of the organism was obtained on the surface of soil extract mannite agar in Petri dishes. These dishes were exposed in duplicate to a mercury arc lamp for varying durations of time.

The soil cultures were prepared in Erlenmayer flasks by inoculating sterilized soil with an emulsion of the organism. These cultures were also incubated for a week to obtain good growth.

At the time of exposure the soil culture was poured in sterile Petri dishes and exposed in duplicate to the ultra violet rays of the mercury arc lamp for 5, 10, 20 or 30 minutes.

The exposures were made at Lahore 90 miles away and the exposed plates were brought to the Laboratory and platings done as soon as possible. The time that elapsed between exposure and plating was about 9 hours, the temperature during this interval varying between 40° and 45°F.

The counts obtained are given below :—

TABLE II

Showing the effect of ultra violet rays on agar and soil cultures

No.	Time of exposure	Dilution	Average count	Total count
		Agar cultures		
1	Control . . .	1 million	4,200	4,200,000,000
2	5 Mts. . . .	40,000	286	11,440,000
3	10 ,, . . .	40,000	95	3,800,000
4	20 ,, . . .	40,000	65	2,600,000
5	30 ,, . . .	40,000	50	2,000,000
		Soil cultures		
1	Control . . .	100 million	51	5,100,000,000
2	5 Mts. . . .	10 ,,	402·5	4,025,000,000
3	10 ,, . . .	1 ,,	1600	1,600,000,000
4	20 ,, . . .	1 ,,	..	Innumerable
5	30 ,, . . .	1 ,,	..	Do.

The above results tend to indicate that the ultra violet rays havenot been able to bring about the death of all the organisms either on the agar surface or in soil cultures. This is clear, however, that the effect of these rays is very injurious, varying directly with the time of the exposure.

The injury is much less serious in soil culture.

Viability of the organism on the seed

(*i*) In order to find out the extent to which the organism could resist desiccation, a quantity of the seed was inoculated and kept stored in a Petri dish in one of the laboratory cupboards.

Sowings were done in triplicate from week to week in sterilized soil pots from this seed. The pots so sown were kept in one of the window spaces away from the direct action of rain, etc. Three control pots were also sown at each interval with sterilized seed. The pots were occasionally watered with sterile tap water. The plants were pulled out after a couple of months and examined for nodule formation.

The results are tabulated below.

Table III

Showing the effect of age on the infective power of an inoculated seed

(Seed inoculated on 4th Nov. 1930)

Sown on	Inoculated nodulation	Control nodulation
4th Nov. 1930 . . .	+ + n. g. . .	— — —
10th Nov. 1930 . . .	+ + n.g. . .	— — —
17th Nov. 1930 . . .	+ + + . .	— — —
24th Nov. 1930 . . .	n.g. n.g. n.g. . .	— — —
1st Dec. 1930 . . .	+ + + . .	— — —
8th Dec. 1930 . . .	+ + + . .	— — —
16th Dec. 1930 . . .	+ + + . .	— — —
23rd Dec. 1930 . . .	+ — — .	— — —
30th Dec. 1930 . . .	— — — . .	— — —

n.g.=No germination.

It is clear from the above that the organism retained its infective power on the seed for about 7 weeks only.

(*ii*) Another experiment was undertaken in the same connection. Different portions of seed were inoculated at an interval of a month and stored in sterile Petri dishes in the laboratory cupboard. Three sterile soil pots were sown from each of these portions on the same day and the nodule formation observed after about a month with the following results :—

Date of sowing	Seed inoculated on	Nodulation
28th Nov. 1931 . . .	11th July 1931 . .	— — —
Do. . . .	7th Aug. 1931 . .	— — —
Do. . . .	7th Sept. 1931 . .	— — —
Do. . . .	8th Oct. 1931 . .	+ + +
Do. . . .	Control . .	— — —

This again shows that the organism retains its infective power on the seed for not more than seven weeks.

Conclusions

In the foregoing experiments some of the cultural characteristics of the organism causing nodules on the roots of berseem (Egyptian clover) have been studied. This organism has been found to be a pseudomonas having its cross inoculant in the organism infecting the roots of *shaftal* (*Trifolium resupinatum*).

According to Löhnis and Hansen [1921] who have divided the bacteria of the leguminous plants into two groups, the representatives of which differ both morphologically and physiologically, the organism infecting the roots of berseem (*Trifolium alexandrinum*) should be peritrichic but inspite of repeated trials we have found this organism to possess only a single polar flagellum.

Cultural characteristics of the organism have been studied on a number of different media. The observations recorded for a change in milk, growth on potato and growth on beef extract media agree with those recorded by Löhnis and Hansen [1921] for the organisms of the clover group.

The pH tolerance of the organism has been found as low as 5.0 on the acid side and 10 or probably a little more on the alkaline side. This agrees with the critical pH limits, recorded by Fred and Davenport [1918].

The thermal death point of the various strains of the organism studied, varied from 52°C. to 54°C. The reason why it can withstand the high summer temperatures of the Punjab (48°C.) is probably due to its being protected in the deeper layers of the soil.

As for the claims made by Thornton and Gangulee [1926] that the nodule organism moves about an inch in 24 hours, the same have not been substantiated by us, in respect of this organism.

Its movement in moist soil has not exceeded half an inch in about a fortnight and that even in a hard pressed soil. This leads one to the conclusion that the organism is transferred in soil more through wind and water than through its organs of locomotion.

The effect of ultra violet rays was studied. The ultra violet light did exercise a destructive effect but this effect varied with the time of exposure. Sun's rays which are so rich in these radiations, owing to the non-permeability of the soil particles to these rays, could have only a partially destructive effect on this organism in soil.

As for the viability of the organism on a seed, it has been found that it retains infective power on the seed for not more than seven weeks.

These results do not agree with those of Fellers [1919] who has found out that the soybean and alfalfa bacteria retain their viability for six to nine months. But his method of storage of the inoculated seed was different from ours.

Fellers [1919] has further mentioned that the largest decrease in the number of organisms on a seed-coat occurs within the first few hours. In view of the fact that there is an exceedingly small percentage of the cells (sometime 1 : 1,000,000) present on a seed that are capable of forming nodules, our results are not extraordinary. The organism investigated is probably more sensitive to desiccation than others.

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Summary

1. The organism producing nodules on the roots of berseem plants is 1 to 3 μ in length and about a μ broad, varying in size at different stages in the life cycle.

2. The organism is mono-trichic with a single polar flagellum.

3. A study of the cultural characteristics of the organism has been made on some of the more important media. The pH tolerance of the organism has been found to be 5 on the acid side and 9·8 or a little more on the alkaline side.

4. The movement of the organism in soil is slow, hardly half an inch in a fortnight.

5. It cross-inoculates with an organism infecting the roots of *shaftal* (*Trifolium resupinatum*).

6. The thermal death point of the organism is between 52°C. and 54°C. But it has been found to survive a temperature of 75°C. in fully grown soil cultures.

7. The organism is sensitive to ultra violet rays. The destructive action of the rays varies with the time of exposure.

8. The organism retains its infective power on the seed, when stored in Petri dishes, for only about seven weeks.

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ABSTRACTS

The method of choosing initial material in breeding (533 : 575 : 578. 08 : 581. 143·26·03). I. A. KOSTJUCENKO (*Semenovodstvo—Seed Growing* 1934 : No. 5 : 18–21).

[*Full summary prepared by the Imperial Bureau of Plant Genetics, Cambridge : Reproduced by kind permission of the Director of the Bureau.*]

In making use of the world collection of varieties for crossing with the local strains the entire success depends on the choice of the most suitable parental varieties. In the past considerable losses of time have been caused by working with unsuitable parental material. Nilsson-Ehle has shewn that to assess the value of varieties it is necessary to grow them under conditions in which they can complete their vegetative cycle. This can be done in Russia only by the method of vernalization. Lyssenko recommends that the best local varieties should be crossed with those world varieties which in the region in question prove earliest after vernalization.

Experiments are described in which various spring wheats were sown in the vernalized and unvernalized state both at Hibiny, with its continuous day, and at Leningrad with its 18-hour day. The cold treatment made hardly any difference to the vegetative period but the long day accelerated it by 3-5 days. This was accompanied by an increase of 0·6-6·6 g. in thousand corn weight which is thus attributed to the effect of the long day in accelerating the second developmental stage, the photostage.

It is necessary therefore to test the parental varieties of long-day plants under continuous illumination to obtain a true picture of their relative merits. For southern regions they will be tested under reduced length of day.

Different plants of a single variety have shewn different reactions to low temperature. Various varieties of winter wheat were vernalized at a temperature of +2°C. for 45, 35, 25 and 20 days. Controls were sown at the same time as the experimental plants. Only 12 of the 60 varieties tested shewed clear response to the cold treatment ; some of these moreover reacted only in the case of treatment for 45 days, others only to the 45 and 35 days treatments, certain others again only to 45, 35 and 25 days. In these two latter groups both winter and semi-winter types were detectable amid the plants which came into ear normally. Similarly again on sowing certain varieties in spring, especially certain Chinese and Japanese varieties, it was observed that some plants came into ear normally whilst others behaved as winter or semi-winter forms, whereas after vernalization treatment these same varieties eared and matured normally and uniformly.

It is clear therefore that many lines which morphologically are pure, with regard to their developmental stages are definitely heterozygous. This heterozygosity is regarded as being partly responsible for the irregular segregations often observed in characters such as yield, earliness, etc. In any case it shews the necessity, in choosing parental forms, of basing conclusions only on the progeny of single plants and not on plants chosen at random from a particular line or variety.

Catalogue number	Origin and name of variety	Variety	Vegetative period from emergence to earing						Reduction in vegetative period in days as a result of increased length of day in the second developmental stage	Thousand corn weight						Increase in thousand corn weight as a result of increased length of day in the second developmental stage.
			Vernalized			Control				Leningrad (18-hour day)			Hibiny (24-hour day)			
			Leningrad (18-hour day)	Hibiny (24-hour day)	Difference	Leningrad (18-hour day)	Hibiny (24-hour day)	Difference		Vernalized	Control	Difference	Vernalized	Control	Difference	
...	U. S. S. R. ' Novinka '	erythrospermum	42	33	9	42	34	8	8	18.3	18.9	0.6	39.1	36.8	2.3	2.3
25725	India, Pusa 52 . .	grecium . .	42	33	9	42	33	9	9	18.4	18.1	0.3	40.3	39.2	0.9	0.6
21105	Asia Minor . .	Reichenbachei	42	35	7	43	35	8	7	16.3	15.3	1.0	37.4	32.6	4.8	3.8
25626	South Africa . .	albidum . .	39	33	6	38	33	5	5	22.0	20.5	1.5	47.7	39.6	8.1	6.6
8085	Canada ' Garnet ' .	lutescens .	39	33	6	39	33	6	6	17.6	17.3	0.3	27.7	32.6	5.1	4.8
25550	Australia . .	alborubrum .	42	34	8	40	34	6	6	17.2	20.7	—3.5	36.5	34.5	2.0	2.0
...	Moscow 2411 . .	erythrospermum	47	41	6	winter	winter	0	6	19.2	...	...	...	...	...	...
14925	U. S. A. ' Nebraska '	erythrospermum	48	41	7	„	„	0	7	20.4	...	...	...	...	...	...

Effect of long day in reducing the vegetative period and increasing the grain weight.

The after harvest ripening of winter wheat in northern regions (633·11 : 581·142) L. TRET' JAKOVA (*Semenovodstvo—Seed Growing* 1934 : No. 6, 33—36).

[*Full Summary prepared by the Imperial Bureau of Plant Genetics, Cambridge : Reproduced by kind permission of the Director of the Bureau.*]

The germinating capacity of the seeds of a number of varieties of winter wheat was tested at varying periods after harvest, from 5 to 50 days. Marked varietal differences were observed, for instance the rye-wheat hybrid and the variety *ferrugineum* 2411 of the Moscow station gave 60-70 per cent germination on the 5th day, rising to 90 per cent on the 35th. Durable and Ukrainka gave a much lower germination on the 5th day and took much longer to reach full germinating capacity—this was attained in the former only on the 45th and in the latter on the 50th day. The variety *erythrospermum* 917 was intermediate between these two types, as were all the other varieties tested.

At another station the rye-wheat hybrid and *ferrugineum* 2411 attained full germinating capacity on the 23rd day whereas Ukrainka was nearly three weeks later. In many localities the date of sowing is past before the majority of the varieties have attained their full germinating capacity.

To study the relation between germination and moisture, the grain of a number of varieties was harvested at different stages of maturity, beginning at the wax-ripe stage, at which all varieties displayed a high germination percentage, which in the rye-wheat hybrid was as much as 98 per cent. Three days later, towards the end of the wax-ripe stage and the beginning of the stage of full maturity, a marked diminution in moisture content was observed in all varieties, falling in some to 10 per cent. At this stage also the rye-wheat hybrid gave the highest germination, though it was lower than at the earlier stage. The changes in germination capacity when compared with those of moisture content were found to be independent. In the stage of complete maturity there is a marked reduction in germination, with only a very small change in moisture content. Later there occurs a parallel rise in germination capacity and fall in moisture content, but even still when all varieties had reached the same moisture content the differences in germination remained quite marked.

Thus some varieties reach a high germinating capacity while retaining a considerable proportion of moisture, whilst others may have a high germination combined with a normal moisture content at a time when their process of maturation is yet incomplete.

The seeds were germinated at 18°C. Germination in river sand invariably gave a higher germination than on filter paper. Chilling for three days at a temperature of 10-13°C. induced rapid germination in unripe seeds which unchilled germinated only very slowly.

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